Functions of mammalian microRNA in innate immunity to microbial infection

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2. Zusammenfassung

MicroRNAs (miRNAs) sind eine Klasse von ~22 nt langen, nicht-kodierenden RNAs, welche mittels Basenpaarung die Translationsrate und Stabilität von mRNAs herabsetzen. In einer Vielzahl von zellphysiologischen Prozessen agieren miRNAs als post-transkriptionale Regulatoren, einschließlich diverser Signaltransduktionswege des angeborenen Immunsystems. Zu Beginn dieser Arbeit stand eine globale Analyse des miRNA Expressionsprofils von mikrobiell infizierten Wirtszellen aus. Die vorliegende Studie beschreibt mittels Hochdurchsatz-Sequenzierung Expressionsveränderungen von miRNAs nach Infektion von kultivierten Wirtszellen mit dem mikrobiellen Modellpathogen *Salmonella enterica* serovar Typhimurium. In Makrophagen, welche zentrale Funktionen in der Orchestrierung der angeborenen Immunität ausüben, wurde im Zuge der Infektion eine Induktion der als immun-assoziiert beschriebenen miRNAs miR-21, miR-146 und miR-155 beobachtet. Überraschenderweise stellten sich darüber hinaus alle Mitglieder der let-7 miRNA Familie in infizierten Makrophagen als herab reguliert heraus. Let-7 miRNAs wurden bislang primär mit der Embryonalentwicklung und terminalen Zelldifferenzierung assoziiert. An dieser Stelle konnte jedoch gezeigt werden, dass let-7 miRNAs die wichtigen Makrophagen-Zytokine IL6 und IL10 post-transkriptional reprimieren. Konsequenterweise bewirkt eine Reduktion der let-7 Expression in mikrobiell aktivierten Makrophagen eine Erhöhung der IL6 und IL10 Produktion. Weiterhin konnten den miRNAs miR-146 und miR-155 wichtige Funktionen in der Steuerung der Sensitivität und Aktivität von Makrophagen gegenüber mikrobiellen Stimuli zugewiesen werden: während miR-146 primär die Aktivität des plasmamembranständigen Lipopolysaccharid-Rezeptors TLR4 herabsetzte und damit einer vorzeitigen inflammatorischen Makrophagenantwort vorbeugte, blieb miR-155 strikt an letztere gekoppelt, um die Aktivität diverser pro-inflammatorischer Signalwege zu begrenzen. Es konnte gezeigt werden, dass eine Stimulation des cytosolischen Immunrezeptors NOD2 eine inflammatorische Makrophagenantwort und die damit einhergehende miR-155 Induktion begünstigt und der negativen Kontrolle durch miR-146 entzieht. Dieses Szenario wird währen der Infektion von Makrophagen mit fakultativ intrazellulären Bakterien wie *S.* Typhimurium relevant und verhindert möglicherweise eine Hyposensitivität gegenüber zellinvasiven Pathogenen. Zusammen legen diese Befunde nahe, dass miRNAs eine wichtige Rolle in der post-transkriptionalen Steuerung der angeborenen Immunantwort auf mikrobielle Pathogene spielen, etwa

durch negative Kontrolle der Wirtszell-Sensitivität bzw. der inflammatorischen Aktivität (miR-146 bzw. miR-155) oder Regulation spezifischer Aspekte der Immunantwort wie der Zytokinexpression (let-7). Diese Erkenntnisse erweitern die Grundlage für künftige Untersuchungen zu Funktionen von miRNAs in der post-transkriptionalen Kontrolle der Wirtsantwort auf bakterielle Pathogene.

3. Summary

MicroRNAs (miRNAs) are a class of ~22 nt long non-coding RNAs that interfere with mRNA translation and stability. In a variety of cell physiological processes miRNAs act as post-transcriptional regulators, including various signal transduction pathways of the innate immune system. At the beginning of this work a global analysis of miRNA expression in microbially infected host cells was missing. Using high-throughput sequencing the present study describes miRNA expression changes upon infection of cultured host cells with the microbial model pathogen *Salmonella enterica* serovar Typhimurium. In macrophages, which exert central functions in the orchestration of innate immunity, the infection caused the induction of known immune-associated miRNAs miR-21, miR-146 and miR-155. Surprisingly, all members of the let-7 miRNA family were down-regulated in infected macrophages. Let-7 miRNAs were primarily associated with functions in embryonic development and terminal cell differentiation previously. This work reports let-7 miRNAs to function in the macrophage inflammatory response by repressing the major cytokines IL6 and IL10 post-transcriptionally. Consequently a reduction of let-7 expression in microbially activated macrophages results in a specific increase in IL6 and IL10 production. Furthermore, miR-146 and miR-155 could be assigned important functions in the control of the sensitivity and activity of macrophages to microbial stimuli: while miR-146 primarily reduced the activity of the plasma membrane associated lipopolysaccharide receptor TLR4, thereby preventing a premature macrophage inflammatory response, miR-155 stayed strictly coupled to inflammation in order to limit the activity of various pro-inflammatory signaling pathways. Interestingly, it could be shown that stimulation of the cytosolic immune receptor NOD2 favors the macrophage inflammatory response and the concomitant induction of miR-155, while bypassing the negative control by miR-146. This becomes relevant during the infection of macrophages with facultative intracellular bacteria such as *S.* Typhimurium and may prevent hyposensitivity to cell-invasive pathogens. Together these findings suggest that miRNAs play an important role in post-transcriptional regulation of the innate immune response to microbial pathogens via negative control of host cell sensitivity and inflammatory activity (miR-146, miR-155) or regulation of specific aspects of the immune response such as cytokine expression (let-7). These findings provide a new basis for further studies of the function of miRNAs in the post-transcriptional control of the host response to bacterial pathogens.

4. Introduction

4.1 Core components of innate defense

4.1.1 Barrier function and origin of the innate immune system

The first line of defense against pathogenic agents consists of the physical barriers provided by the skin and the epithelial surfaces that are continuous with it, such as the respiratory or gastrointestinal epithelia. As these inner epithelia are far more vulnerable than the rigid, keratinized skin they constitute primary sites of infection by microbial and viral pathogens. As a countermeasure the surfaces of the gastrointestinal, respiratory and urogenital tracts are covered by mucus, a thick layer of fluid that is rich in glycoproteins and antimicrobial peptides. Augmenting the anti-pathogenic capacity of this antiseptic first barrier, mucosal surfaces are rich in cells of the innate immune system. Cellular innate immunity largely relies on the recognition of pathogen associated molecular patterns (PAMPs) through evolutionarily conserved pattern recognition receptors (PRRs), primarily by macrophages and granulocytes. Moreover, cells missing self-antigens or exposing stress markers may be eradicated by natural killer (NK) cells. This enables a rapid response to a pre-defined array of foreign antigens or abnormal cell surfaces. Furthermore, mast cells and dendritic cells exert important functions in the bridging of innate and adaptive immune responses (see below and section 4.3.5).

All immune cells derive from hematopoietic stem cells (HSCs). During embryogenesis HSCs at first reside in hepatic tissue compartments before translocation into the developing bone marrow niche (Fig.1), which constitutes the primary hematopoietic compartment for the remainder of a mammal's life span. According to the classical model (Lai and Kondo, 2008; Orelio and Dzierzak, 2007; Seita and Weissman, 2010) HCSs give rise to multipotent progenitors that do not have self-renewing potential. These differentiate into common lymphoid progenitors or common myeloid progenitors (Fig. 1). The common lymphoid progenitors generate the lymphoid lineage comprising mainly B and T cells that confer adaptive immunity and the above mentioned NK cells (Fig. 1). By contrast, the common myeloid progenitors give rise to megakaryocyte/erythrocyte precursors which differentiate into platelets and erythrocytes and into the common granulocyte/macrophage precursors. The latter give

rise to granulocytes (neutrophils, eosinophils and basophils) and monocytes, which may differentiate into macrophages and dendritic cells (DCs), (Fig. 1).

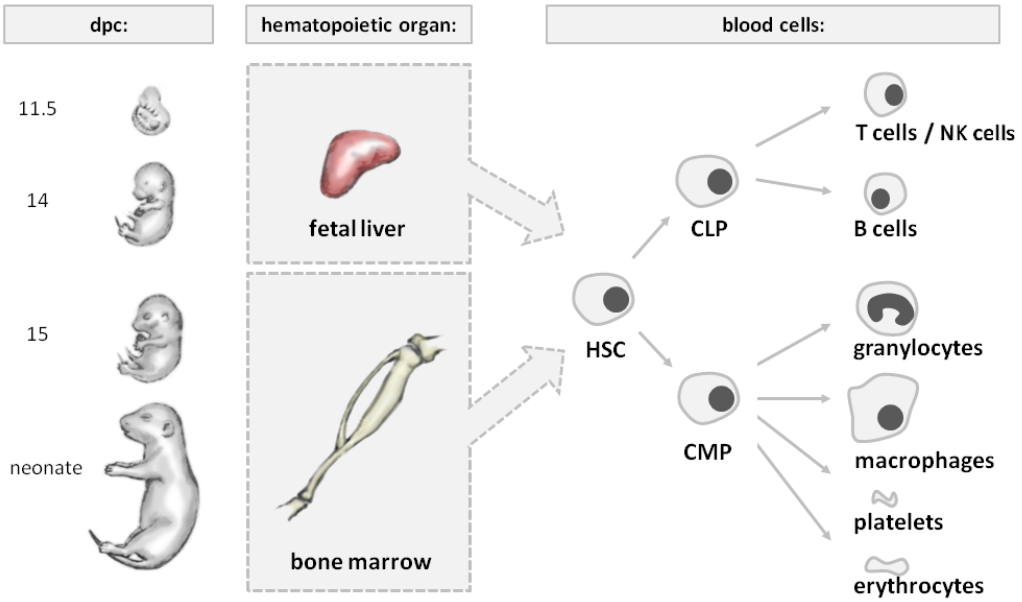

Figure 1: Simplified scheme of hematopoiesis in embryonic and post-natal mouse development. The age of the embryo in dpc (days post coitus) is indicated to the very left, followed by the predominant hematopoietic organ. Irrespective of the site of hematopoiesis, blood cells derive from a population of self-renewing hematopoietic stem cells (HSC). These give rise to common lymphoid progenitor (CLP) or common myeloid progenitor (CMP) populations. Terminally differentiated cells of the lymphoid lineage (T cells, NK cells, B cells) eventually arise from the CLP pool whereas the mature cells of the myeloid lineage (granulocytes, macrophages, platelets and erythrocytes) arise from the CMP population. Adapted from Orelio and Dzierzak (2007) and emouseatlas.org.

4.1.2 Primary functions of innate immune cells in defense against pathogens

Ligation of PAMPs to highly specific PRRs on the surface or in the cytoplasm of host cells constitutes a primary mode of innate defense activation. A prototypic example is the recognition of the gram-negative bacterial cell wall component lipopolysaccharide (LPS) by Toll-like receptor 4 (TLR4) which is conserved from insects to mammals (Silverman and Maniatis, 2001). TLR4 is a representative of a larger class of trans-membrane immune receptors (the Toll-like receptors) that sense a variety of PAMPs and typically trigger cellular pathways that activate inflammation master-regulators such as the IRF or NFκB transcription factors (see 4.3.2). TLR4 is expressed on a variety of immune- and non-immune-cells and its activation by LPS initiates a cell-type specific defense program

(O'Connell, et al., 2005). In macrophages for instance this involves the production of toxic metabolites (such as nitric oxide), an increase of the cellular phagocytosis rate and phagosomal acidification, or initiation of pyrogen production (i.e. prostaglandin E2). Furthermore, TLR4 stimulation of macrophages initiates the production of chemokines and cytokines that recruit immune cells from the periphery and function as immune hormones, respectively (Fig. 2).

Among the cells that are rapidly recruited by macrophages are mast cells which are activated by PRR agonists or by antigen-ligation to immunoglobulin of the IgE isotype that covers the cell surface (Abraham and St John, 2010). Upon activation mast cells cause vasodilatation and elevate blood-vessel and tissue permeability mainly through histamine secretion, thereby facilitating the infiltration of infected tissue by other classes of professional immune cells (Fig. 2) such as granulocytes (Karasuyama, et al., 2010; Nathan, 2006). Neutrophils for instance are rapidly recruited to the site of inflammation and function as short lived effector cells that kill pathogenic agents such as bacteria upon ingestion through the production of reactive oxygen species (ROS) and through release of antimicrobial peptides such as cathelicidin, cathepsin or defensins (Fig. 2). Eosinophils are another class of granulocytes that are recruited to sites of acute infection to release toxic metabolites such as ROS or toxic proteins such as major basic protein (MBP). Basophils are the third and also the least abundant class of granulocytes. Similar to mast cells basophils are coated by immunoglobulin of the IgE isotype and have been implicated in histamine and heparin release which locally increases tissue perfusion.

Finally, NK cells continuously probe for surface expression of self- and stress-markers (Lanier, 2005). Self markers provide an inhibitory signal to NK cells while the absence of self-markers or the presence of stress-markers may induce killing of target cells via release of cytolytic granules from NK cells. Thereby, NK cells assist in detection and removal of infected or transformed cells. NK cell activity may be modulated by accessory factors such as macrophage cytokines (Fig. 2), (Newman and Riley, 2007).

In summary, the soluble and cellular components of the innate immune system establish a rapidly responding first line of defense that impedes the breaching of the environmentally exposed epithelia as well as the subsequent systemic dissemination of pathogens in the host organism. As depicted in Fig. 2, among the diverse types of innate immune cells macrophages play a key role in the orchestration of innate defense. Recently, microRNAs (miRNAs) have been shown to regulate key gene expression programs of innate immune cells at the post-transcriptional level, as delineated below.

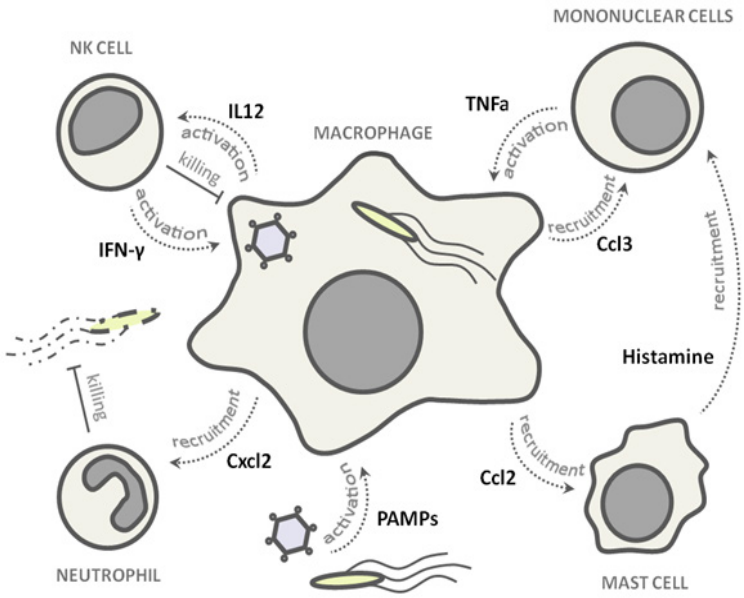

Figure 2: Schematic representation of innate immune orchestration by macrophages. Activation of macrophages by PAMPs (pathogen associated molecular patterns) initiates an innate inflammatory response that involves the production of cytokines such as the NK cell activating interleukin IL12. NK cells in turn may give an activating signal or kill macrophages, if infected or extensively stressed. A number of different macrophage chemokines recruits other types of innate immune cells such as neutrophils, which participate in pathogen eradication by phagocytosis and production of antiseptic metabolites. Furthermore, mast cells are recruited to the site of inflammation and via histamine-induced vasodilatation facilitate the further recruitment of mononuclear cells to the inflammatory hot-spot (such as granulocytes, monocytes, lymphocytes), which may in turn sustain the inflammatory response via macrophage activating cytokines such as TNFα. Cytokines and recruiting factors depicted in this figure are representative examples.

4.2 MicroRNA

4.2.1 The discovery of microRNA and RNA interference

MicroRNAs (miRNAs) constitute a class of small noncoding RNAs of ~ 22 nucleotides in size that act as post-transcriptional regulators of gene expression in many eukaryotes. MiRNAs were first described in 1993 when the short RNA lin-4 was reported to regulate lin-14 mRNA translation in early larval stage progression of the nematode model organism *Caenorhabditis elegans* (Lee, et al., 1993). The second miRNA to be reported was let-7 which had been identified as a regulator of *C. elegans* late larval stage progression via control of lin-42 mRNA translation (Reinhart, et al., 2000). Furthermore,

the let-7 miRNA sequence was found to be conserved from worms to humans (Pasquinelli, et al., 2000) and soon miRNAs were recognized as a functional class of deeply conserved small noncoding RNA in metazoa (Lagos-Quintana, et al., 2001; Lau, et al., 2001; Lee and Ambros, 2001). A robust understanding of the basic principles of miRNA function emerged in the years following the discovery of the RNA interference (RNAi) pathway, which induces decay of a given cellular RNA upon ectopic delivery of a short double-stranded RNA harboring a target-RNA complementary strand. First indication for RNA-induced gene silencing derived from a report published in 1984, describing the modulation of thymidine kinase mRNA expression by microinjection of anti-sense ssRNA expression constructs into cultured mammalian cells (Izant and Weintraub, 1984). This work stimulated further research in this direction (Melton, 1985; Weintraub, et al., 1985), yet it took more than another decade until Craig Mellow and co-workers revealed that highly potent and specific RNA-based interference with *C. elegans* gene expression could be achieved by ectopic administration of short dsRNA, which proved to be far more effective and long lasting than ssRNA (Fire, et al., 1998). Soon after, endogenous small anti-sense RNA was reported to confer post-transcriptional silencing of transgene or virus RNA in plants (Hamilton and Baulcombe, 1999). These reports provided the basis for the subsequent decoding of the RNAi and miRNA pathway. In the years following the discovery of RNAi the molecular machinery driving targeted transcript repression via exogenous or endogenous dsRNA and miRNA was characterized. The molecular machinery driving miRNA biogenesis and function via components of the RNAi pathway is delineated in detail in the following section. Much of the work that contributed to our present understanding of the miRNA machinery has been performed in model organisms such as *C. elegans* or *Drosophila melanogaster*. As the present work aims to foster a better understanding of miRNA function in innate immunity to microbial infection of mammals the following sections will focus on the mammalian homologs of the miRNA pathway if not specified otherwise.

4.2.2 MicroRNA biogenesis and function

MiRNAs originate from RNA-polymerase II dependent protein-coding or non-coding transcripts (pri-miRNAs) that are co-transcriptionally processed by the nuclear RNaseIII enzyme[1] Drosha. The latter is recruited to ~ 70 nt long RNA-hairpin structures within

[1] RNase III enzymes constitute a conserved class of endonucleases that recognize and cleave dsRNA

the pri-miRNA through interaction with the dsRNA binding protein DGCR8. The resulting protein-complex, also referred to as the Microprocessor complex, mediates endonucleolytic release of the RNA hairpin that is then termed the pre-miRNA (Fig.3). The nascent pri-miRNA transcript upon Drosha cleavage has been reported to associate with the 5'-3' exonuclease XRN2 which may catch up with RNA-polymerase II to terminate transcription (Ballarino, et al., 2009). This mechanism of pri-miRNA termination however may account only for a subfraction of pri-miRNA transcripts since the majority of miRNAs seems to derive from spliced introns (Morlando, et al., 2008). In these cases, direct interaction of the Microprocessor complex with splice factors and acceleration of intron decay through XRN2 upon pre-miRNA release has been proposed to elevate splicing efficiency (Morlando, et al., 2008; Shiohama, et al., 2007) rather than leading to transcriptional termination. Thus miRNAs may originate from spliced transcripts without interfering with nuclear mRNA or long non-coding RNA maturation pathways, while non-spliced pri-miRNA transcripts seem to be co-transcriptionally degraded upon pre-miRNA release. Finally, a miRNA biogenesis pathway has been reported in which spliced and debranched introns fold into pre-miRNAs independent of the Microprocessor complex. These so called mirtrons then enter the subsequent steps of the regular miRNA maturation pathway (Ruby, et al., 2007).

The pre-miRNA hairpin in the nucleus associates with the nuclear export receptor exportin-5 to be transported through the nuclear pore complex in a RanGTP dependent manner (Fig. 3), (Lund, et al., 2004; Yi, et al., 2003). Exportin-5 binds the pre-miRNA only in the presence of a 3' overhang that derives from Drosha cleavage, thus mediating selective export of only the correctly processed RNA (Zeng and Cullen, 2004).

Upon translocation into the cytosol the pre-miRNA associates with the RNaseIII enzyme Dicer and the endonuclease Ago2, assisted by the dsRNA binding protein TRBP (Chendrimada, et al., 2005; Gregory, et al., 2005; Liu, et al., 2004). Dicer functions to cleave off the terminal loop of the pre-miRNA hairpin (Bernstein, et al., 2001; Hutvagner, et al., 2001; Lee, et al., 2003), resulting in an RNA duplex that dissociates from Dicer and TRBP (Fig. 3). Similar to pre-miRNA biogenesis in the nucleus, exceptions exist for the maturation of miRNA in the cytosol: a rare pathway has been reported that utilizes the endonucleolytic activity of Ago2 to process the miR-451 precursor independent of Dicer (Cifuentes, et al., 2010). Regardless of the initial pathway however, the resulting RNA duplex is subsequently unwound by the activity of several helicases, assisted by Ago2 (Winter, et al., 2009). Typically, the strand with the lower thermodynamic stability at its

5’ base pair stays associated with Ago2 and becomes incorporated into the miRNA induced silencing complex (miRISC), which mediates mRNA binding and repression (Fig. 3), (Schwarz, et al., 2003). The miRISC incorporated miRNA duplex strand is also termed the guide strand since it mediates sequence-specific binding of the miRISC complex to mRNA targets, whereas the opposite strand, also referred to as the passenger strand, is degraded upon unwinding.

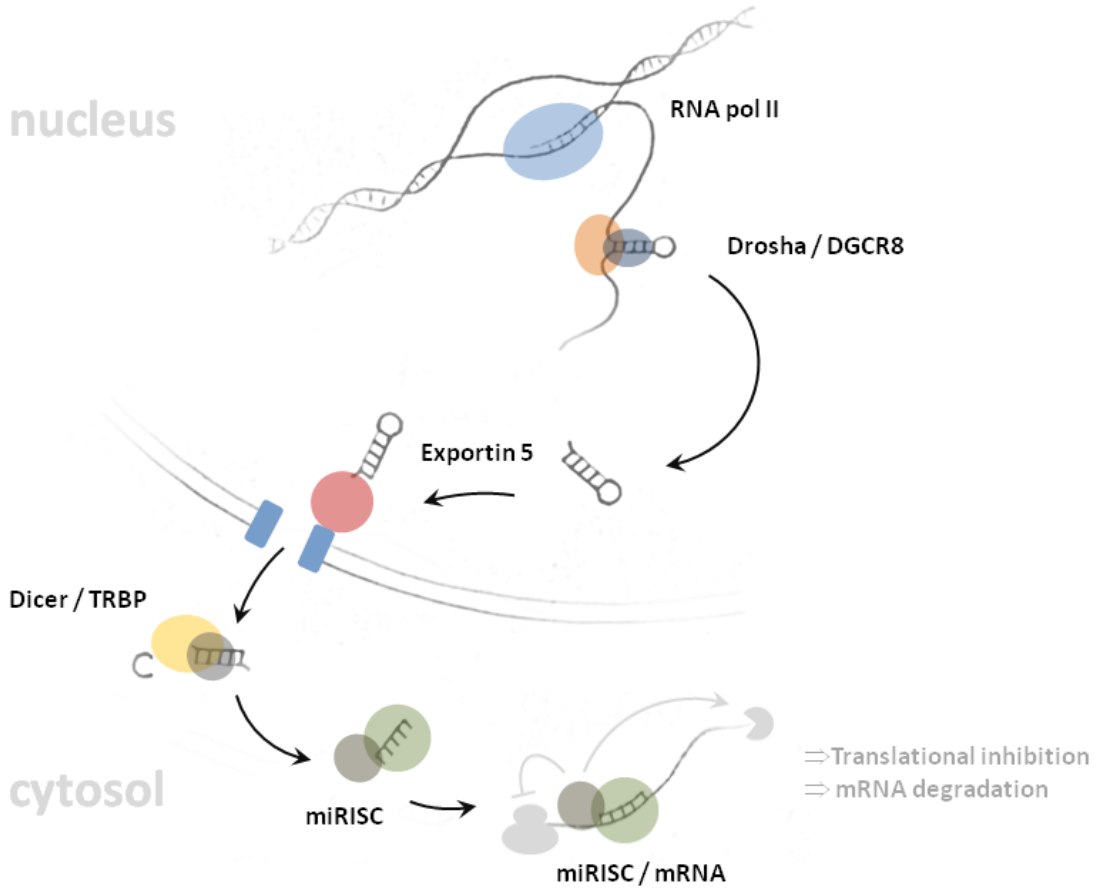

Figure 3: Simplified scheme of the mammalian miRNA biogenesis pathway. RNA polymerase II (RNA pol II) generates the primary miRNA transcript (pri-miRNA) which locally folds into an RNA hairpin structure. The hairpin (pre-miRNA) is cleaved out by the endonuclease Drosha with the aid of the dsRNA binding protein DGCR8. Exportin 5 mediates translocation of the pre-miRNA into the cytosol where the terminal loop is cleaved off by Dicer, assisted by the dsRNA binding protein TRBP. The miRNA guide strand is incorporated into the Ago2 and TNRC6 containing miRISC complex whereas the passenger strand is degraded. miRNA-guided binding of miRISC to mRNA through base-pair complementarity within the miRNA “seed“ region mediates translational inhibition and / or mRNA decay.

The key components of the miRISC complex are Ago2 and TNRC6 (also referred to as GW182). The three-dimensional structure of a mature miRNA (the guide strand) in complex with the Ago2 protein revealed that only RNA bases 2-8 are constantly exposed

for efficient hydrogen bond formation with complementary mRNA sequences (Jinek and Doudna, 2009). This correlates well with the observation that a 6 to 8 nucleotide stretch starting at 5' position 2 of the miRNA (also referred to as the seed sequence) is critical for target mRNA repression (Brennecke, et al., 2005; Doench and Sharp, 2004). Typically, base pairing of the miRNA with its target mRNA involves some additional positions downstream of the seed sequence as well. Upon miRNA-Ago2 mediated mRNA binding, usually within the 3'UTR, the second basal component of the miRISC complex, TNRC6, initiates translational repression of the targeted transcript. This involves recruitment of the CCR4-NOT1 deadenylase complex that initiates mRNA degradation and inhibits translational initiation (Hafner, et al., 2011).

It has remained under debate until very recently whether the primary mode of miRNA action in mammals is translational inhibition, target mRNA degradation or both. While in plants miRNAs seem to predominantly cause Ago2-mediated target mRNA cleavage and subsequent decay due to full complementarity with the target sequence, the seed-restricted complementarity in animals is believed to protect from mRNA cleavage. Recent ribosome profiling data suggest that nevertheless target mRNA decay and translational inhibition typically occur at similar rates upon miRNA binding in mammals (Guo, et al., 2010); however, exceptions may still exist where translational inhibition is the primary mode of target repression (Bazzini, et al., 2012). A single miRNA may control hundreds of mRNA targets (Fabian, et al., 2010; Selbach, et al., 2008), thus adding another layer of complexity to eukaryotic gene expression programs. To date miRNAs have been implicated in diverse basal aspects of animal biology such as embryonic development, cellular proliferation or maintenance of adult stem- and germ-cell pools, and in higher body functions such as the shaping of neural networks or immune responses to pathogens and cancer cells (Lakshmipathy, et al., 2010; Loya, et al., 2010; O'Connell, et al., 2012; Pauli, et al., 2011).

4.3 MicroRNA directed control of innate immune responses

4.3.1 Requirement of the miRISC pathway in innate immunity

Evidence for a critical role of miRNA in immunity arose from knockout studies concerning the essential miRNA biogenesis factor Dicer. Ablation of both Dicer alleles led to early abrogation of fetal development at 7.5 – 8.5 days post coitum (dpc) in mice,

demonstrating a critical role of the miRNA pathway in embryogenesis (Bernstein, et al., 2003). Conditional Dicer ablation in the lymphoid lineage of immune cells blocked progression of bone marrow B cell development into the mature peripheral stages (Koralov, et al., 2008; Ventura, et al., 2008). Conditional knockout of Dicer in murine granulocyte-macrophage progenitors was reported to result in neonate lethality shortly after birth, probably due to a lung morphological defect (Alemdehy, et al., 2012). When fetal liver cells of those embryos were transplanted into irradiated mice to circumvent the neonate lethality, the myeloid lineage developed, yet with a significant decrease in peripheral macrophage and neutrophil numbers. This probably involves repression of apoptosis pathways by miR-125 which is elevated in adult compared to embryonic HSCs and ectopic expression of miR-125 in embryonic HSCs significantly expanded the pools of peripheral circulating myeloid cells (Bousquet, et al., 2010; Guo, et al., 2010; Ooi, et al., 2010). In summary, Dicer, and probably miRNA, is essential for proper development of the lymphoid lineage and regulation of myeloid cell peripheral survival and proliferation.

4.3.2 MicroRNA in myeloid innate immunity

Pioneering work identified miR-146 and miR-155 as induced miRNAs in monocytes upon stimulation with microbial or viral PAMPs such as microbial TLR4 ligand LPS or viral dsRNA analog polyI:C, which is sensed by the TLR3 (Taganov, et al., 2006). Both miRNAs are evolutionarily conserved among vertebrates (Heimberg, et al., 2008) and are co-induced during the macrophage inflammatory response to LPS in order to feedback-control TLR4 signal transduction (Quinn and O'Neill, 2011). Briefly, macrophage TLR4 signaling is relayed by the intracellular TLR adaptors MyD88 and TRIF which initiate two distinct signaling cascades. The TRIF dependent cascade activates the transcription factors IRF3 and IRF7 which trigger the expression of type I interferons (type I IFNs) that play a major role in anti-viral defense. The MyD88 arm of TLR4 signal transduction on the other hand constitutes the main pathway to activation of the NFκB transcription factor downstream of TLR4. NFκB in turn not only activates the expression of mediators of inflammation such as cytokines, inducible enzymes or acute phase proteins but also of miRNAs such as miR-146 and miR-155 (Quinn and O'Neill, 2011). Of note, activation of miR-146 and miR-155 in macrophages is not restricted to TLR4 but also occurs downstream of other TLRs and upon stimulation of cellular receptors of pro-

inflammatory cytokines such as IL-1β or TNFα (O'Connell, et al., 2007; Taganov, et al., 2006).

Upon NFκB dependent activation, miR-146 targets the key signal transducers of the MyD88 dependent TLR pathway, IRAK1 and TRAF6, thereby establishing negative feedback control the of macrophage TLR4 response (Taganov, et al., 2006). Consequently, homozygous miR-146 knockout mice are hypersensitive to intravenous administration of LPS compared to their wild-type littermates (Zhao, et al., 2011). Furthermore, monocytes and macrophages pre-treated with TLR4 ligand LPS may enter a state of cross-tolerance to secondary stimulation with TLR ligands, also referred to as endotoxin-tolerance, which probably serves to protect from inflammation induced pathologies (Biswas and Lopez-Collazo, 2009). The maintenance of endotoxin-tolerance has been reported to involve negative control of TLR signaling by miR-146 as well (Nahid, et al., 2009; Nahid, et al., 2011).

Similar to miR-146, miR-155 also feedback represses TLR signal transduction. Specifically, miR-155 targets the TLR signal transducer TAB2, which also plays a central role in cytokine signaling, indicating that miR-155 might feed-back control both TLR and cytokine induced macrophage responses (Ceppi, et al., 2009). Paradoxically, in addition to negative feedback control of PRR and cytokine signaling, miR-155 has been shown to repress SHIP-1 which acts as a negative modulator of TLR induced NFκB activity in macrophages (O'Connell, et al., 2009). Moreover, miR-155 has been demonstrated to stabilize the messenger of pro-inflammatory cytokine TNFα (Thai, et al., 2007; Tili, et al., 2007) and to target SOCS1, a negative regulator of type I IFN expression (Wang, et al., 2010). These reports demonstrate that miR-155 may also be involved in the initiation of pro-inflammatory responses, which seems to contradict its reported functions in negative control of TLR and cytokine signaling. MiR-155 has therefore been suggested to function as an initial activator of the macrophage inflammatory response via SHIP-1 and SOCS1 repression while preventing the response from getting out of control by negative feed-back regulation of TLR and cytokine receptor signaling (O'Neill, et al., 2011).

Another miRNA that is induced in macrophages in response to NFκB activation is miR-21. Unlike miR-146 and miR-155, miR-21 does not function via direct feedback suppression of core components of TLR and cytokine receptor signal transduction. Rather miR-21 targets the tumor suppressor PDCD4 (Sheedy, et al., 2009), which through a yet unknown pathway promotes NFκB activation. Negative control of PDCD4

by miR-21 reduces pro-inflammatory gene expression in favor of anti-inflammatory cytokine IL10 expression (Sheedy, et al., 2009).

Two other miRNAs, miR-9 and miR-132, have been observed to be regulated in LPS challenged monocytes (Bazzoni, et al., 2009; Taganov, et al., 2006). In activated monocytes miR-132 suppress the transcriptional co-activator of interferon responsive genes, p300. Expression of the miR-132 gene in turn depends on p300 co-activation, which suggests another miRNA-directed negative feedback loop (Lagos, et al., 2010). The LPS responsiveness of miR-132 was revealed by an independent miRNA profiling study in activated monocytes and neutrophils (Bazzoni, et al., 2009). In addition the authors report induction of miR-9 through TLR-MyD88 dependent signaling upon LPS challenge of both monocytes and neutrophils. Interestingly miR-9 was found to negatively regulate the expression of p105 a precursor to the core NFκB subunit p50 (Bazzoni, et al., 2009), providing yet another example of miRNA-directed negative feedback control of inflammation.

4.3.3 MicroRNA in lymphoid innate immunity

MiRNAs have also been implicated in effector functions of the lymphoid lineage derived NK cells. NK cells vitally participate in the anti-viral response by killing virally infected host cells and augment anti-microbial defense pathways via release of the cytokine interferon-γ (IFN-γ). Expression profiling revealed that mature NK cells express ~300 miRNAs including specific markers of cells of the lymphoid lineage such as miR-150 (Fehniger, et al., 2010). Impairment of miRNA biogenesis by conditional knockout of Dicer revealed a significant decrease in interleukin-15 (IL15) stimulated survival (Sullivan, et al., 2012). IL15 is primarily produced by activated monocytes or macrophages and promotes local NK-cell activity (Carson, et al., 1997). Moreover, miRNA deficient NK cells appeared to produce abnormally high levels of IFN-γ, major functions of which are to promote macrophage lysosomal activity, ROS production and antigen presentation. IFN-γ hyper-production by Dicer deficient NK cells could be traced back to a loss of expression of the miR-15 family which targets the 3'UTR of the IFN-γ messenger (Sullivan, et al., 2012). Importantly, activated NK cells in mice infected with microbial pathogens *Listeria monocytogenes* and *Mycobacterium bovis* were found to down-regulate miR-29, which targets IFN-γ as well (Ma, et al., 2011). Similar to macrophages, NK cell activation also involves induction of miR-155, which augments inflammatory

gene expression, including cytokine IFN-γ production, via suppression of SHIP-1 (Trotta, et al., 2012). Collectively, these reports suggest that production of the major NK cell cytokine IFN-γ is simultaneously controlled by multiple miRNAs.

4.3.4 MicroRNA in epithelial innate immunity

MiRNAs have been implicated in the post-transcriptional control of innate inflammatory gene expression programs of non-immune cells as well. Cholangiocytes, the epithelial cells of the bile duct, constitute a well-established model in epithelial innate immunity and have been extensively characterized with respect to their miRNA repertoire. For instance, challenge of cholangiocytes with the microbial TLR4 ligand LPS reduces the expression of miRNAs of the let-7 family (Chen, et al., 2007; Hu, et al., 2009). As let-7 miRNAs target TLR4, decreased let-7 expression elevates TLR4 surface expression (Chen, et al., 2007). Furthermore, let-7 miRNAs target CIS, a negative regulator of pro-inflammatory cytokine signaling. Thus let-7 down-regulation limits cholangiocyte inflammatory gene expression induced by autocrine/paracrine cytokine signaling (Hu, et al., 2009).

Treatment of alveolar epithelial cells with the pro-inflammatory cytokine IL1β has been reported to induce miR-146, which functions to limit epithelial cytokine IL8 expression (Perry, et al., 2008). Elevated miR-146 expression in neonate intestinal epithelial cells prevents premature immune responses to the establishing commensal microflora via repression of TLR4 signal transducer IRAK1 (Chassin, et al., 2010), a target of miR-146 in macrophages (see above).

Furthermore, miRNAs have been implicated in the control of immune-relevant surface adhesion molecule expression in epithelial cells. Cholangiocyte stimulation with T_{h1} cell / NK cell cytokine IFN-γ for instance down-regulates miR-221 expression to relieve repression of ICAM-1, a cell surface adhesion molecule that enhances the interaction with T cells (Hu, et al., 2010). Moreover, IFN-γ treated cholangiocytes down-regulate miR-513 expression to relieve repression of PD-L1, a transmembrane protein that functions to inhibit spontaneous T cell activation upon docking, thereby strengthening the specificity of adaptive immune responses (Gong, et al., 2009).

4.3.5 MicroRNA at the interface of innate and adaptive immunity

Although the innate immune system confers powerful and rapid defense against invading pathogens it may fail to protect from infections as novel virulence strategies continuously evolve. In order to escape the innate immune response pathogens may reduce PAMP exposure, express aberrant PAMP variants or actively subvert host intra- and intercellular signaling (Hornef, et al., 2002). As innate immunity may not adapt to changing PAMP structures or virulence strategies during an individual's life-span the adaptive immune system serves to provide acquired protection against pathogens. Rather than simply providing a secondary line of defense however, the adaptive immune system assists in innate responses and vice versa.

Dendritic cells constitute an immune cell type that functions to bridge innate and adaptive immunity. Similar to macrophages, dendritic cells may originate from monocytes and are activated by PAMP or cytokine stimuli. At the site of infection, however, dendritic cells may pick up pathogenic material and translocate it into the lymph node germinal centers where pathogen-derived antigens are presented to naïve $CD4^+$ T cells via cell surface MHC-II. Activated $CD4^+$ T cells in turn differentiate into effector and memory $CD4^+$ T cells. The relatively short-lived effector T cells (T helper [T_h]) may release cytokines that promote cellular immunity and specifically phagocyte activity (T_{h1} $CD4^+$ cells) or co-activate B cells that recognize the same antigen to promote humoral immunity (T_{h2} $CD4^+$ cells), (Romagnani, 2000). Dendritic cell assisted activation of B and T cell immunity in the lymph nodes is referred to as the germinal center response. Interestingly, upon homozygous ablation of the miR-155 host gene BIC mice failed to be immunized against *Salmonella enterica* serovar Typhimurium, likely due to impaired antigen presenting capacity by dendritic cells (Rodriguez, et al., 2007). Furthermore a supportive role of miR-155 in germinal center formation was reported that involved positive regulation of TNFα expression (Thai, et al., 2007).

As part of the germinal center response B cells undergo class-switching to produce immunoglobulin of different isotypes. The IgE isotype for instance is bound by the surface Fc-receptor of mast cells, a cell type that closely interacts with cells of the innate immune system (section 4.1.2). Ligation of a matching antigen to surface IgE typically provokes mast cell activation. Expression profiling of IgE activated mast cells revealed the induction of miR-132, which in turn was found to control the release of HB-EGF, a growth factor involved in cutanous wound healing (Molnar, et al., 2012).

Besides their critical roles in the initiation of adaptive immunity dendritic cells respond to innate immune stimuli similar to macrophages. This is also reflected in the repertoire of miRNAs that are employed in the response to PAMP stimulation, involving for instance the induction of miR-155 (Bai, et al., 2012; Ceppi, et al., 2009). In contrast to macrophages however, miR-146 seems to be rather constitutively expressed in dendritic cells to prevent from inappropriate activation of pro-inflammatory responses (Jurkin, et al., 2010). Moreover, PAMP stimulated dendritic cell activation entails negative feedback control of TLR4 and pro-inflammatory cytokine production by miR-511 and miR-142 (Sun, et al., 2010; Tserel, et al., 2011).

4.3.6 Overview of microRNA-mediated control in the innate immune system

Collectively, post-transcriptional control by miRNA seems to be employed throughout the innate immune system to adjust the activities of major immune-related pathways. Table 1 summarizes the specific implications of miRNAs in innate immunity in different types of cells as described in the foregoing sections.

Tabele 1: Major functions of miRNAs in innate immune homeostasis

MiRNA	Proposed function	Reference
	Monocytes, macrophages, dendritic cells	
miR-9	Induced in PAMP-challenged monocytes; negatively regulates **NFκB** via p105/p50 sub-unit	Bazzoni, et al., 2009
miR-21	Induced in PAMP-challenged monocytes and macrophages; suppresses **NFkB** activator PDCD4	Sheedy, et al., 2009
miR-132	Induced in PAMP-challenged monocytes; suppresses **interferon response** by targeting of p300	Bazzoni, et al., 2009; Lagos, et al., 2010; Taganov, et al., 2006
miR-142	Induced in PAMP-challenged dendritic cells; negatively regulates **IL6** production	Sun, et al., 2010
miR-146	Induced in PAMP-challenged monocytes and macrophages and constitutively expressed in dendritic cells; negatively controls **TLR signaling**	Taganov, et al., 2006, Zhao, et al., 2011
miR-155	Induced in PAMP-challenged monocytes, macrophages and dendritic cells; **sensitizes cells** to inflammatory stimuli, promotes **TNFα** production, negatively controls **TLR and cytokine signaling**	Ceppi, et al., 2009; O'Connell, et al., 2009; Thai, et al., 2007; Wang, et al., 2010
miR-511	Induced in PAMP-challenged dendritic cells; negatively regulates **TLR4**	Tserel, et al., 2011
	Granulocytes	
miR-9	Induced in LPS-challenged neutrophils; negatively regulates **NFκB** via p105/p50	Bazzoni, et al., 2009

miR-132	Induced in LPS-challenged neutrophils; suppresses **interferon response** by targeting of p300	Bazzoni, et al., 2009
NK cells		
miR-15, miR-16	Steady state expressed; limit **IFN-γ** expression	Sullivan, et al., 2012
miR-29	Down-regulated upon NK cell activation: relieves repression of **IFN-γ** directly	Ma, et al., 2011
miR-155	Induced upon activation; promotes **IFN-γ** production via SHIP-1 suppression	Trotta, et al., 2012
Epithelial cells		
let-7 miRNAs	Down-regulated upon PAMP stimulation of epithelial cells; relieve **TLR4** suppression and promote negative control of **cytokine-signaling**	Chen, et al., 2007; Hu, et al., 2009
miR-146	Induced upon PAMP- and cytokine-stimulation of epithelial cells; represses **TLR4 signaling** and **IL8** expression	Perry, et al., 2008; Chassin, et al., 2010
miR-221	Down-regulated upon cytokine-stimulation of epithelial cells; promotes **T cell adhesion** via relieve of ICAM1 expression	Hu, et al., 2010
miR-513	Down-regulated upon cytokine-stimulation of epithelial cells; inhibits spontaneous **T cell activation** via relieve of PD-L1 surface marker expression	Gong, et al., 2009

4.4 MicroRNA in infection

4.4.1 MicroRNA in viral infection

Manipulation and usage of the miRNA pathway by DNA viruses. Reports on miRNA functions in the response of eukaryotic host cells to infection were limited to virus infection studies until very recently. DNA viruses extensively manipulate the host miRNA pathway as part of their virulence strategies. The molecular toolkit of these viruses ranges from suppressors of RNAi to transactivators, repressors or functional mimics (orthologs) of host miRNAs. A majority of examined dsDNA viruses of the herpesviridae family encodes miRNAs (Cullen, 2011; Pfeffer, et al., 2005), with Eppstein-Barr virus (EBV) being the most well studied representative. During the early stage of infection EBV stimulates proliferation of infected B cells and replicates. As soon as the infection is controlled by the adaptive immune response EBV switches into a latency stage (latency stage III) that allows it to persist predominantly in memory B cells (Amon and Farell, 2004). So far EBV has been found to express 40 miRNAs (Cosmopoulos, et al., 2009). These miRNAs function to inhibit the host antiviral response, for instance via suppression of the expression of T cell attracting chemokine CXCL11 (Xia, et al., 2008) or of stress marker MICB that would promote killing by NK cells (Nachmani, et al., 2009).

Furthermore, EBV miRNAs promote survival of infected cells via repression of apoptosis-mediators PUMA and BIM (Choy, et al., 2008; Marquitz, et al., 2011). Interestingly the viral protein LMP1, which itself is under the control of several EBV miRNAs (Lo, et al., 2007) triggers NFκB dependent expression of miR-155, a ubiquitous regulator of innate and adaptive immunity in the host (section 4.3), (Gatto, et al., 2008). This propels transformation of infected B cells through the anti-apoptotic properties of miR-155. Consequently, EBV infection may result in the development of malignant B cell lymphomas (Kuppers, 2003; Linnstaedt, et al., 2010). In summary, EBV does not only utilize its self-encoded repertoire of miRNAs to manipulate host gene expression but also employs host miRNA miR-155 to achieve B cell transformation and to successfully establish long-term infection.

Interestingly, dsDNA viruses other than EBV have been described to encode functional orthologs of host miRNA miR-155. Similar to EBV, Kaposi's sarcoma-associated herpesvirus (KSHV) infects B cells and induces pro-survival pathways, what may result in the development of B cell lymphomas (Keller, et al., 2000). In contrast to EBV however, KSHV encodes its own functional ortholog of miR-155, miR-K12-11 (Boss, et al., 2011; Skalsky, et al., 2007). MiR-K12-11 also assists in immune evasion by suppression of the IKKε kinase that mediates initiation of the anti-viral type-I interferon response (Liang, et al., 2011). Furthermore KSHV benefits from PAMP-induced activation of host miRNAs miR-146 and miR-132, as inhibition of both miRNAs not only fuels the host cell inflammatory response but also inhibits viral replication (Lagos, et al., 2010). In summary, the major DNA virus models KSHV and EBV establish stable host infection by the aid of both host and self-encoded miRNAs.

A striking virulence strategy of a DNA virus that concerns the miRNA pathway is applied by herpesvirus saimiri (HVS), which causes T cell lymphomas in New World primates. HVS expresses two miRNA-sequestering ncRNAs that degrade host miRNAs miR-16 and miR-27 via coupled decay (Cazalla and Steitz, 2010). Host miR-16 and miR-27 are normally expressed at steady state levels and their degradation by HVS promotes virus replication through a pathway that is yet to be revealed. Importantly, however, this example suggests that steady-state expressed host miRNAs may antagonize viral replication unless targeted for decay by the pathogen.

Manipulation and utilization of the miRNA pathway by RNA viruses. RNA viruses were predicted not to encode miRNAs to avoid from processing and fragmentation of their genomes by the miRNA machinery (Cullen, 2011). Yet, bovine leukemia virus (BLV) was recently reported to encode miRNAs. This retrovirus encodes a cluster of miRNAs that is inert to processing by Drosha (Kincaid, et al., 2012); instead, RNA polymerase III dependent transcription produces short (~70 nt) RNAs that are processed further by Dicer to enter the miRISC pathway. BLV in its natural host induces B cell lymphomas. Intriguingly, one of its miRNAs is an ortholog of miR-29, which is implicated in development of lymphocytic leukemia and also in control of interferon production (Ma, et al., 2011; Santanam, et al., 2010). Other RNA viruses are known to modulate or utilize the miRNA pathway. Hepatitis C virus (HCV) replicates within hepatocytes that naturally express high levels of miR-122. This miRNA binds to the 5' end of the viral RNA genome and enhances its translation and replication through mechanism that is not yet understood (Jopling, et al., 2005). Inhibition of miR-122 by an antisense oligonucleotide reduces virus replication and titers in infected chimpanzees (Lanford, et al., 2010) and a phase II clinical trial is currently evaluating the efficacy of a miR-122 inhibitor in human HCV therapy[2].

4.4.2 MicroRNA in bacterial infection

Similar to viruses, pathogenic bacteria extensively manipulate host cellular signaling pathways to cope with the host immune response. A new, intriguing example of subversion of host defense by microbes comes from the plant field. The gram-negative plant pathogenic bacterium *Pseudomonas syringae* translocates microbial effector proteins into the host cell to antagonize accumulation of mature miRNAs associated with plant antimicrobial defense (Navarro, et al., 2008). Colonization of *Arabidopsis thaliana* leafs by *P. syringae* triggers the flagellin sensor FLS2 which mediates activation of miR-393 transcription. This miRNA represses the auxin signaling pathway, which negatively regulates plant anti-microbial defense. Translocated *P. syringae* effectors, however, subvert host defense, likely by repressing miR-393 accumulation. This demonstrates the ability of gram-negative bacteria to manipulate host defense via specific repressors of

2 Santaris Pharma press release (11.05.2011): "Santaris Pharma A/S Phase 2a data of miravirsen shows dose-dependent, prolonged viral reduction of 2-3 logs HCV RNA after four-week treatment in Hepatitis C patients"

the miRNA pathway, similar to what has been described for mammalian viruses previously. In the context of animal infections however, manipulation of the host miRNA pathway by bacterial effector proteins still lacks experimental proof. Yet, many microbial pathogens reportedly manipulate host immune related pathways to evade defense by secreting specific effector proteins into the host cell (section 4.5).

At the onset of the present work reports on miRNA expression changes in mammalian host cells upon live microbial infection were still missing. Yet, induction of miR-146 and miR-155 in monocytes treated with the purified *Salmonella enterica* or *Escherichia coli* PAMP LPS had been reported (Taganov, et al., 2006). However, many mammalian pathogens are cell invasive, which may entail stimulation of cytosolic PRRs that mount a host response that is different from extracellular challenge with LPS (section 4.5). Furthermore, as exemplified by *P. syringae*, the host miRNA response may be subverted during actual host infection by the action of bacterial virulence factors. These considerations motivated the analysis of miRNA expression changes in response to a mammalian cell-invasive microbial model pathogen.

4.5 The model pathogen *Salmonella enterica* serovar Typhimurium

4.5.1 *Salmonella enterica* pathogenicity

Salmonella enterica serovar Typhimurium (henceforth *S.* Typhimurium) is a gram-negative, facultative intracellular microbial pathogen and representative of the enterobacteriaceae. This family of bacteria comprises many major pathogens such as *Vibrio cholerae*, *Yersinia pestis* or *Shigella flexneri*. *S.* Typhimurium constitutes a leading cause of enteric disease in a broad range of animal hosts. Both the genetic virulence determinants of *S.* Typhimurium and the host immune response that it elicits have been studied in depth. The pathogen infects its mammalian hosts primarily via contaminated vegetable or the fecal-oral transmission route and causes either gastro-intestinal or systemic infections, depending on the host species. While in humans *S.* Typhimurium primarily infects cells of the intestinal epithelium and causes foodborne gastroenteritis, in mice it rather disseminates into inner organs via infection of sub-mucosal macrophages and may induce lethal typhoid fever (Haraga, et al., 2008). This resembles human infections with the closely related but human-specific pathogen *Salmonella enterica* serovar Typhi (henceforth *S*. Typhi), which causes systemic bacteremia and

typhoid fever (Santos, et al., 2003). Experimental mouse infection with *S.* Typhimurium therefore constitutes a major model for human *S.* Typhi infection.

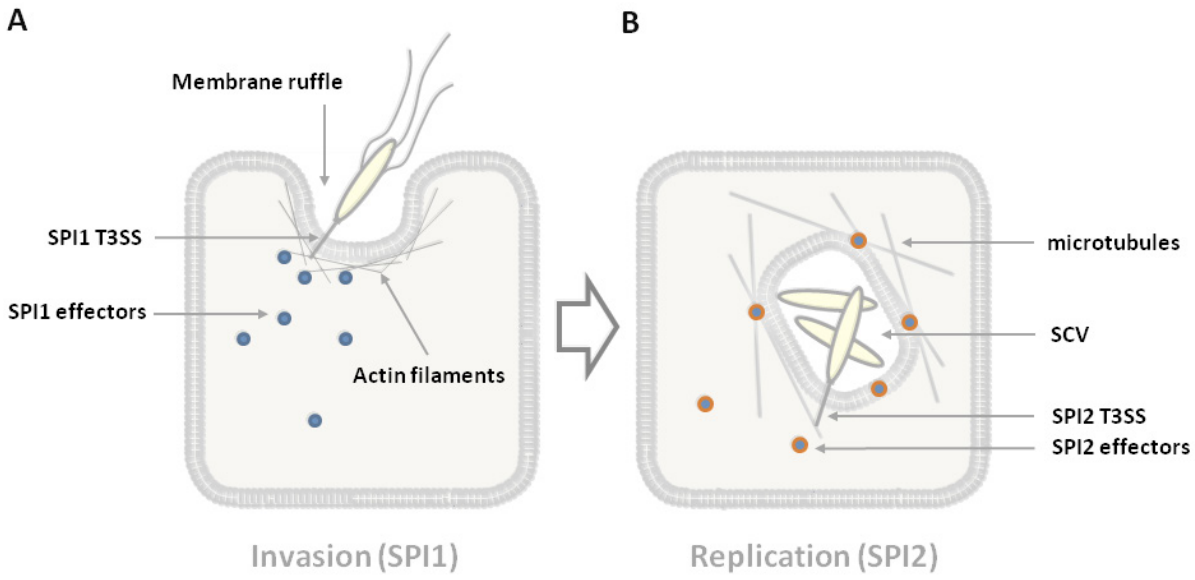

Figure 4: Invasion of host cells and intracellular replication by *S.* Typhimurium. Via the SPI1 encoded T3SS and effector proteins *S.* Typhimurium induces host membrane ruffles via actin cytoskeleton rearrangements, thereby inducing its phagosomal uptake (A). Specific SPI1 effectors inhibit phago-lysosome formation thereby preventing from intracellular bacterial killing. TLR-induced phagosomal acidification is sensed by *S.* Typhimurium and triggers expression of the SPI2 T3SS and its associated effectors. The SPI2 effectors remodel the *S.* Typhimurium containing phagosomal compartement (B) to establish the *Salmonella* Containing Vacuole (SCV), which is attached to the microtubules of the host cytoskeleton and is protected from being redirected to cellular vesicular trafficking pathways. The SCV allows *S.* Typhimurium to persist and replicate intracellularly for up to several days.

Common to gram-negative microbial pathogens is the utilization of protein secretion systems that serve to translocate microbial effector proteins into the host cell. Loss of the secretion systems typically confers strong attenuation of microbial virulence. *S.* Typhimurium encodes two major secretion systems (the type 3 secretion systems, T3SSs) in two distinct genomic regions referred to as the *Salmonella* Pathogenicity Island 1 and 2 (SPI1 and SPI2). Each island encodes a number of secreted effector proteins that subvert host defense pathways and facilitate host cell invasion and intracellular replication. Upon ingestion, *S.* Typhimurium in the murine host enters the gut-associated lymphoid tissue underneath the intestinal epithelial cell layer (Broz, et al., 2012). This primarily involves transcytosis through M-cells, which function to translocate foreign material to the lymphoid tissues that are rich in immune cells. Here,

S. Typhimurium may invade monocytes and macrophages and disseminate into inner organs such as liver and spleen via the blood stream.

Cell invasion requires the SPI1 T3SS and its associated effector proteins, which promote host cytoskeletal rearrangements to facilitate pathogen entry (Fig. 4). Entry occurs mainly via phagosomal uptake. The acidified phagosomal environment triggers expression of the SPI2 encoded T3SS and its associated effector proteins (Arpaia, et al., 2011), which promote the establishment of the *Salmonella* Containing Vacuole (SCV). The SCV is a phagosome derived vesicle that is protected from lysosomal fusion and eludes the host cell vesicular trafficking pathways. Thereby the SCV serves as a microenvironment allowing the bacterium to survive and replicate intracellularly (Fig. 4). Aside from their primary functions in cell entry and intracellular replication, *S*. Typhimurium SPI1 and SPI2 effectors subvert host inflammatory and cell-death associated pathways (section 4.5.3), (Srikanth, et al., 2011).

4.5.2 The host response to *Salmonella enterica* serovar Typhimurium

The immune response to *S.* Typhimurium involves various PRR-PAMP interactions. Major *S.* Typhimurium immune ligands are lipoprotein, LPS and flagellin, which activate the plasmamembrane receptors TLR2, TLR4 and TLR5 respectively and so activate inflammatory gene expression programs (Kawai and Akira, 2006). This may entail activation of macrophage chemokines that recruit accessory immune cells (section 4.1.2) or cytokines such as IL6 or TNFα that propel the inflammatory response. In intestinal epithelial cells TLR5 activates IL8, which is involved in the recruitment of innate immune cells to mucosal tissue (Gewirtz, et al., 2001). Aside from this first line of defense mammalian host cells may also sense microbial PAMPs via TLRs that are integrated into the phagosomal membranes. This involves for instance sensing of bacterial unmethylated DNA via vesicular TLR9, which triggers inflammatory gene expression similar to TLR2, TLR4 and TLR5 (Kawai and Akira, 2006). Interestingly, while animals lacking TLR2 and TLR4 fail to control and clear the infection (Seibert, et al., 2010), simultaneous deletion of TLR2, TLR4 and TLR9 renders mice less susceptible to *S.* Typhimurium (Arpaia, et al., 2011). In fact *S.* Typhimurium requires phagosome acidification to switch on SPI2 gene expression and this phagosomal acidification requires TLR signaling (Arpaia, et al., 2011). This demonstrates that TLRs play an important role in the defense against *S.* Typhimurium but may also provide stimuli for the bacterium to activate virulence gene expression programs.

Aside of this intricate host-pathogen interplay at the level of the TLR response the facultative intracellular life-style of *S.* Typhimurium also entails the activation of cytosolic PRRs other than TLRs. For instance, the flagellin protein FliC is sensed not only by the plasmamembrane receptor TLR5 but also by the cytosolic PRR NLRC4 (Miao, et al., 2010_b). This receptor belongs to the recently identified family of Nucleotide Oligomerization Domain like receptors (NOD-like receptors or NLRs). Another known agonist of NLRC4 is the T3SS protein PrgI. Receptor activation by either FliC or PrgI activates a caspase-1 dependent rapid cell death pathway that is referred to as pyroptosis (Miao, et al., 2010_a; Miao, et al., 2010_b). This exposes *S.* Typhimurium to neutrophils in the surrounding that may eliminate the bacterium (Miao, et al., 2010_a). On the other hand *S.* Typhimurium may benefit from rapid host cell death and release into the extracellular environment by disseminating and starting new rounds of host cell invasion, intracellular replication and release (Mastroeni, et al., 2009). Another function of caspase-1 aside from its roles in cell death is the proteolytic cleavage of the protein-precursors of major cytokines IL1β and IL18. IL1β is a major pro-inflammatory cytokine that triggers production of pyrogens, augments cellular inflammatory responses and fuels production of further systemically acting pro-inflammatory cytokines such as IL6 (Dinarello, 2009). IL18 on the other hand may stimulate T_{h1} adaptive immune responses and IFN-γ dependent intestinal inflammation (Sims and Smith, 2010). In line with the important functions of caspase-1 in pyroptosis and activation of inflammatory cytokine production, caspase-1 knockout renders mice hypersusceptible to systemic *S.* Typhimurium infection (Lara-Tejero, et al., 2006; Raupach, et al., 2006). Yet, NLRC4 is not the only caspase-1 inducing NLR triggered by *S.* Typhimurium, in line with NLRC4 knockout mice being not significantly hypersusceptible to *S.* Typhimurium induced pathologies (Raupach, et al., 2006). Instead, caspase-1 may be activated by a second NLR, NLRP3, and knockout of both NLRP3 and NLRC4 renders mice hypersusceptible to *S.* Typhimurium, similar to caspase-1 knockout mice (Broz, et al., 2010). However, the *S.* Typhimurium PAMP that activates NLRP3 is yet to be characterized.

Similar to NLRC4 and NLRP3, another pair of NLRs has been reported to play redundant roles in antimicrobial defense: NOD1 and NOD2 (a.k.a. NLRC1, NLRC2) initiate NFκB dependent inflammatory gene expression programs upon sensing of cytosolic microbial peptidoglycans. Importantly, NOD1/2 dependent NFκB activation is mediated by different signaling components than TLR dependent NFκB activation (Kersse, et al., 2011). This suggests an alternative, TLR downstream layer of defense that

is activated when plasma- or phagosome-membranes have been breached by cell invasive microbial pathogens. Similar to TLRs, members of the emerging family of NLRs are critically involved in host defense against *S.* Typhimurium but might as well contribute to disease pathology and dissemination of the bacterium (Homer, et al., 2012; Keestra, et al., 2011; Miao, et al., 2010_a).

4.5.3 Subversion of host innate immunity by *Salmonella enterica* serovar Typhimurium

As *S.* Typhimurium represents an extensively studied model pathogen that triggers both extra- and intracellular innate immune pathways in a variety of host cell types, purified *S.* Typhimurium agonists serve as a standard in the field of PRR research and were used in the pioneering research on miRNAs in innate immunity (Taganov, et al., 2006). In contrast to stimulations with purified PRR ligands however, reports on miRNA in live microbial infection of mammalian cells were still missing at the onset of this study. Yet, *S.* Typhimurium may extensively manipulate host cellular signaling pathways. During host cell entry a battery of SPI-1 effector proteins, including SipA, SipC, SopE, SopE2 and SopB, subvert actin polymerization pathways to induce cytoscelletal rearrangements leading to a ruffling of the host plasmamembrane (McGhie, et al., 2009). These ruffles promote S. Typhimurium phagosomal uptake. Host Rho-GTPases that are triggered by SopE, SopE2 and SopB to propell actin polymerization also activate the NFκB pathway and thereby the inflammatory response (Bruno, et al., 2009). Furthermore, Rho-GTPases activated by SopE may trigger NLRC4 to promote IL1β and IL18 dependent gut inflammation (Muller, et al., 2009). On the other hand, SPI-1 effector protein, AvrA, has been demonstrated to inhibit NFkB pathway activation (Jones, et al., 2008) and inflammation. Inhibition of NFκB activity is also conferred by the SPI2 effectors SseL and SspH and the effector GogB, which is translocated by both the SPI1 and the SPI2 T3SS. (Le Negrate, et al., 2008; Rohde, et al., 2007) This suggests that *S.* Typhimurium may both promote and inhibit host inflammatory responses. Upon host cell invasion *S*. Typhimurium employs a battery of SPI2 encoded effectors that promote the maintenance of the SCV, a vacuole that protects the bacterium from intracellular defense pathways such as autophagy or lysosomal detoxification (Agbor and McCormick, 2011; Broz, et al., 2012). In summary, *S*. Typhimurium may manipulate host innate defense via secrteted effector proteins, which may both inhibit and activate inflammatory responses. Many of the above depicted studies on subversion of host cellular pathways by *S*. Typhimurium secreted effectors have been conducted using *in*

vitro models of human epithelial cells and murine macrophages, such as Hela and RAW264.7 cells. The present work addresses the roles of miRNAs in innate immunity to microbial infection using these well-established cellular models of *S*. Typhimurium interaction with its hosts.

5. Aim of this study

Many cell physiological processes are regulated at the post-transcriptional level by miRNAs, including immune reactions to pathogenic material. Yet, a global analysis of host miRNA expression changes upon live microbial infection of mammalian cells was missing at the onset of this study. Many major microbial pathogens have a facultative intracellular life-style, subvert key host cellular pathways and stimulate cytosolic PRRs. This suggests that the host miRNA response to live microbial infection may differ considerably from challenge with purified TLR ligands such as LPS. Using high-throughput sequencing of small RNAs the present study aims to determine host miRNA expression changes globally in mammalian host cells challenged with the well-established facultative intracellular microbial pathogen model *Salmonella enterica* serovar Typhimurium. As *S*. Typhimurium extensively manipulates host cellular immune pathways this study aims to assess whether and how expression levels of defense associated miRNAs are manipulated by the pathogen. On the other hand, the present work also inspects the involvement of host innate immune pathways in the regulation of miRNAs upon *S*. Typhimurium infection. Moreover, comparison of miRNA expression in established human and murine infection models seeks to identify potenital common denominators in the host miRNA response to *S*. Typhimurium. Finally, this study aims to dissect the functions of miRNAs in the host response to *S*. Typhimurium to contribute to a better understanding of the roles of miRNAs in innate immunity to live microbial infection.

6. Results

6.1 Profiling of microRNA expression in *S.* Typhimurium-infected host cells

For miRNA expression profiling murine RAW264.7 macrophages and Hela229 human epithelial cells were selected as they constitute extensively studied *in vitro* models of *S.* Typhimurium infection (section 4.5.3). Expression changes of miRNAs were determined globally by high-throughput sequencing of cDNA libraries generated from the small RNA fraction (19-34 nt) of wild-type *S.* Typhimurium infected cells (4 and 24 h pi) or non-infected cells (0 h and 24 h mock treatment). An average number of ~50.000 cDNAs was sequenced per library with ~70% of the sequences in each library mapping to miRNAs annotated in miRBase version 14.0 (Griffiths-Jones, et al., 2008).

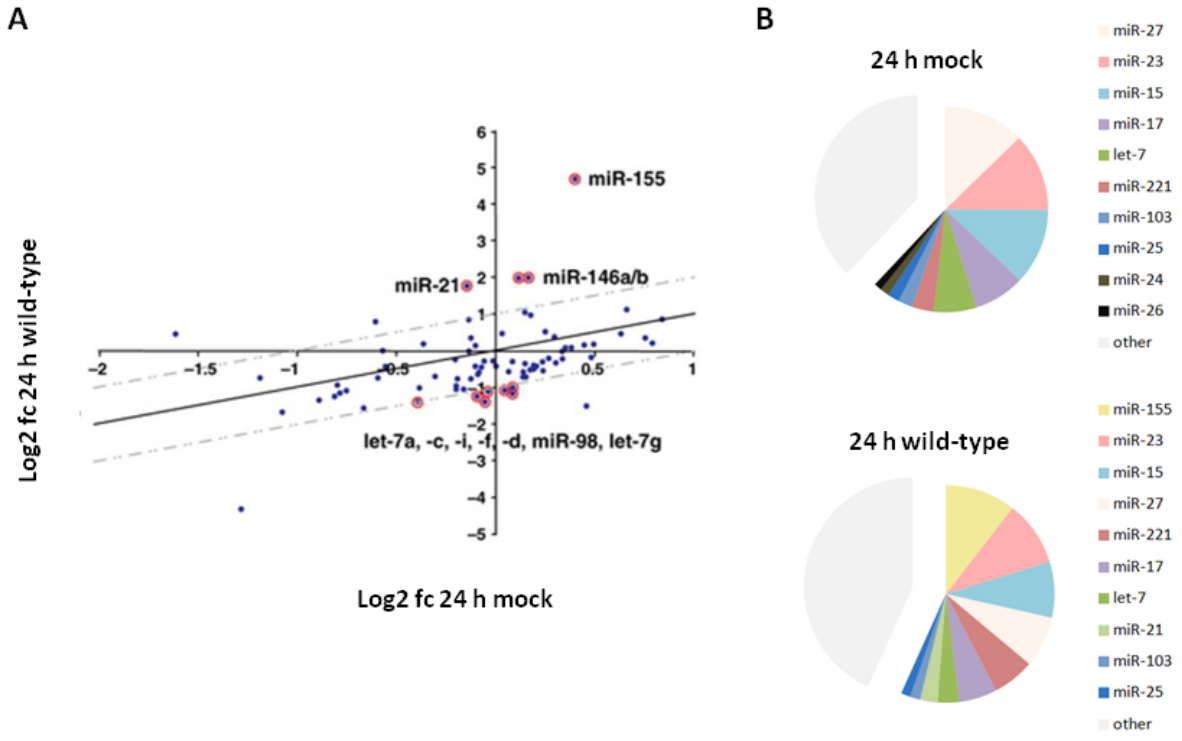

Figure 5: High-throughput sequencing reveals miRNA expression changes in *S.* Typhimurium infected RAW264.7 macrophages. (A) Log2 miRNA fold-changes comparing 0 h to 24 h control treatment (x-axis) and 0 h control treatment to 24 h wild-type *S.* Typhimurium infection (y-axis). Continuous line indicates linear correlation between infection and control-treatment; dashed lines indicate a 2-fold difference comparing control- and infection-specific regulations. **(B)** Abundance of the 10 top-expressed miRNA-families in the indicated sequencing datasets, expressed as % of all reads. Light grey slice indicates residual reads remaining to 100 %, respectively.

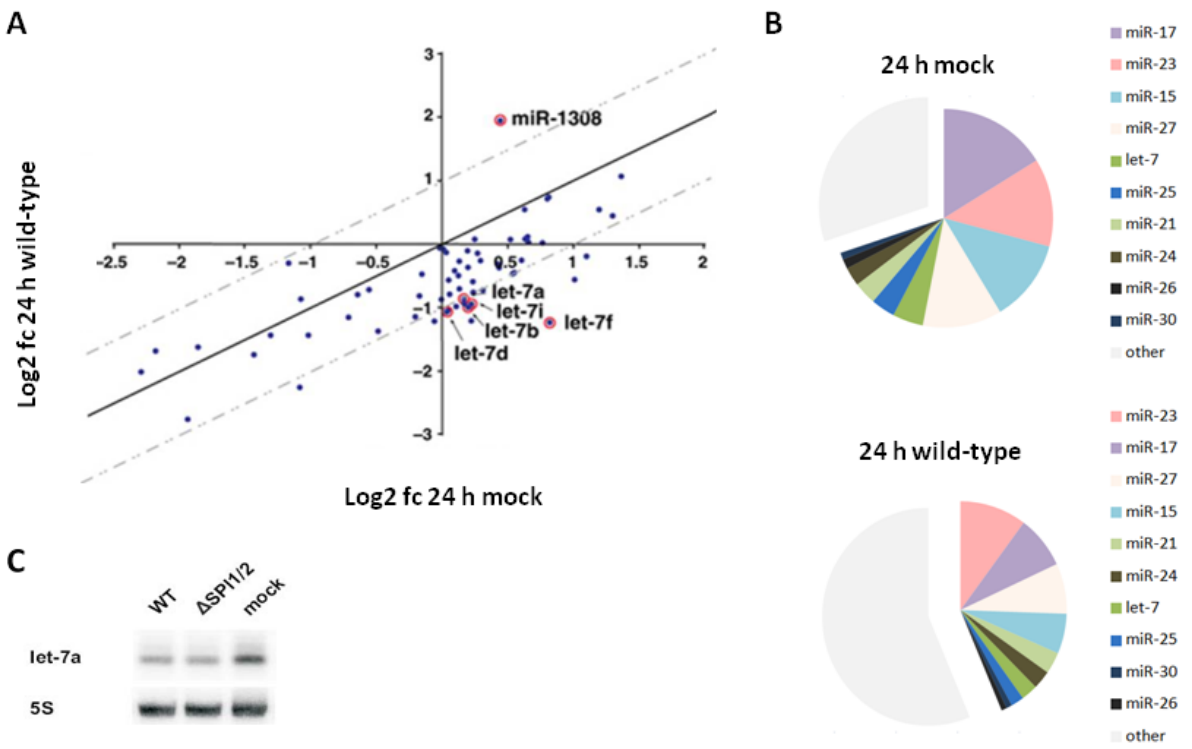

Figure 6: High-throughput sequencing reveales miRNA expression changes in *S.* Typhimurium infected Hela229 cells. (A,B) For description of data-representation see legend of Fig. 5. **(C)** Northern blot analysis of let-7a expression in RAW264.7 cells challenged with wild-type (WT) or ΔSPI1/2 *S.* Typhimurium for 24 h. 5S rRNA serves as a reference control.

MiRNAs expression changes in RAW264.7 macrophages and Hela229 cells were determined by comparing relative abundances of the cDNA reads in the infection and control datasets. Murine macrophages and human epithelial cells exhibited similar profiles of abundant miRNAs, with the majority of sequencing reads mapping to miRNAs of the miR-27, miR-23, miR-15, miR-17 and let-7 families in the 24 h control libraries (Fig. 5B, Fig. 6B). In RAW264.7 macrophages high-throughput sequencing revealed induction of miR-21 (3.4-fold), miR-146a (4-fold), miR-146b (4-fold) and miR-155 (25.7-fold) 24 h post *S.* Typhimurium infection compared to mock treatment (Fig. 5A). These miRNAs had previously been reported to be up-regulated upon LPS stimulation of monocytes (Taganov, et al., 2006). Intriguingly, a ~2-fold down-regulation of all expressed members of the let-7 family of miRNAs (let-7a,-c, -d, -f, -g, -i, miR-98) was observed in *S.* Typhimurium infected RAW264.7 macrophages (Fig. 5A). Whereas miR-21, miR-146 and miR-155 were unregulated in Hela229 cells, again all members of the let-7 miRNA family were down-regulated by ~2-fold 24 h post infection (pi) compared to mock-treatment (Fig. 6A). Northern blot analysis confirmed this regulation upon challenge with wild-type but also with ΔSPI1/2 *S.* Typhimurium (Fig. 6C). The only

relevantly induced miRNA in *S.* Typhimurium infected Hela229 cells was miR-1308 (Fig. 6A). Hela229 cDNA reads containing the annotated 18 nt long miR-1308 sequence were typically expanded by another 8 nts towards the 3' end. Blasting these full-length reads against the human reference genome (ENSEMBL) they were found to align four times within a region of ~35 kb in size on human chromosome 1 (miRBase version 14 annotation for the miR-1308 locus is on chromosome X). This region was marked by elevated GC content (Fig. 7A), which may be indicative of horizontally acquired elements (Kijima and Innan, 2010).

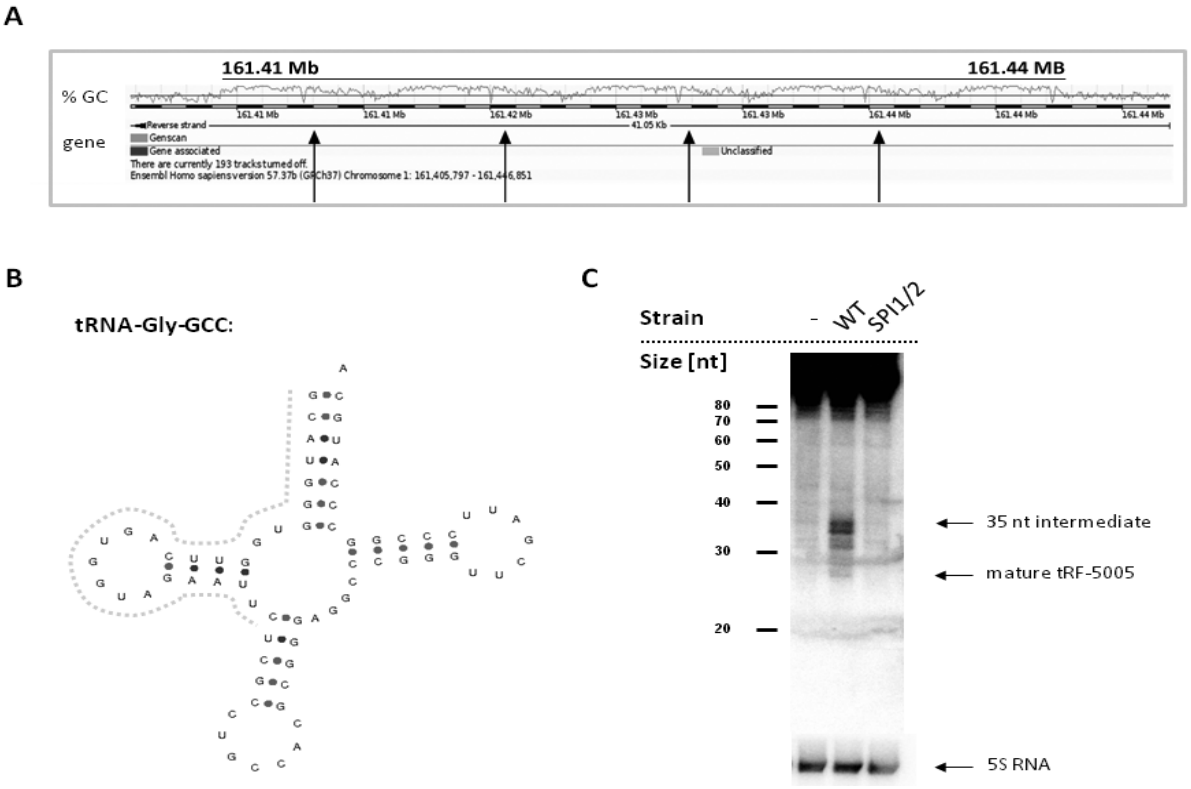

Figure 7: miR-1308 is a tRNA derived fragment. (A) Mapping of the 26 nt long sequence inferred from miR-1308 cDNA sequencing reads to the human reference genome (ENSEMBL BlastN result). Arrows indicate the positions of the blast hits on chromosome 1. **(B)** Predicted secondary structure of tRNA-Gly-GCC (tRNA-scan SE database) matching to the repeated sequence extracted from (A) that contains the miR-1308 cDNA blast hits. Dashed line marks the corresponding cDNA read sequence detected in the sequencing experiment depicted in Fig. 6. **(C)** Northern blot analysis of the miR-1308-corresponding RNA in 24 h control-treated Hela229 cells (lane 1) or cells infected for 24 h with wild-type (lane 2) or mutant *S.* Typhimurium lacking the SPI1 and SPI2 virulence systems (lane 3).

On closer inspection the four sequence hits marked the 5' end of a 71 nt long repeated sequence element. As this is a typical size frame of tRNAs the sequence was blasted against the "tRNA-scan SE" database (http://lowelab.ucsc.edu/tRNAscan-SE/) and was

identified as tRNA-Glycin-GCC (Fig. 7B). Northern blot analysis using a nucleotide-probe complementary to the first 18 nts of the tRNA 5' end revealed that wild-type *S. Typhimurium* infection induces ~26 and ~35 nt processing fragments (Fig. 7C). Processing of tRNA has been reported previously (Cole, et al., 2009; Lee, et al., 2009) and the 26 nt fragment of tRNA Gly-GCC has been referred to as tRF-5005 (Lee, et al., 2009). Therefore miR-1308 likely represents a tRNA-derived fragment and not a miRNA.[3]

Collectively, the here presented expression data confirm the previously known induction of miRNAs miR-21, miR-146a/b and miR-155 in the monocyte/macrophage inflammatory response; yet, dismissing miR-1308 as a miRNA, no induction of miRNAs is observed in the epithelial cell clone in response to microbial challenge. The down-regulation of let-7 miRNA expression appears as a common denominator of macrophages and epithelial cells challenged with *S*. Typhimurium.

6.2 Triggers of *S.* Typhimurium induced microRNA expression changes

Macrophages are key players in the orchestration of host innate immunity and their response to microbes down to the level of individual PRR-PAMP interactions has been studied extensively (section 4.1.2). While purified *Salmonella enterica* LPS was known to elicit specific miRNA expression changes (Taganov, et al., 2006) it remained to be investigated whether live *S.* Typhimurium manipulates the PAMP induced miRNA response. To this end the miRNA regulation profile obtained from wild-type *S.* Typhimurium infected macrophages (Fig. 5) was compared to miRNA regulation patterns in macrophages challenged with *S.* Typhimurium SPI mutants (see section 4.5) lacking the SPI1 region (ΔSPI1 strain), the SPI2 region (ΔSPI2 strain) or both (ΔSPI1/2 strain). Recovery of colony forming units (CFUs) from macrophages challenged with the different mutants confirmed the expected virulence defects (Fig. 8A). That is, the ΔSPI1 strain was severely impaired in macrophage entry compared to the wild-type strain (4 h pi), yet replicated when taken up (24 h pi). The ΔSPI2 strain entered macrophages normally compared to wild-type infection (4 h pi) but failed to survive and replicate intracellularly (24 h pi). The ΔSPI1/2 strain as expected was impaired both in entry and intracellular survival (4 h and 24 h pi).

[3] miR-1308 has meanwhile been discontinued in the main miRNA reference database (miRBase.org)

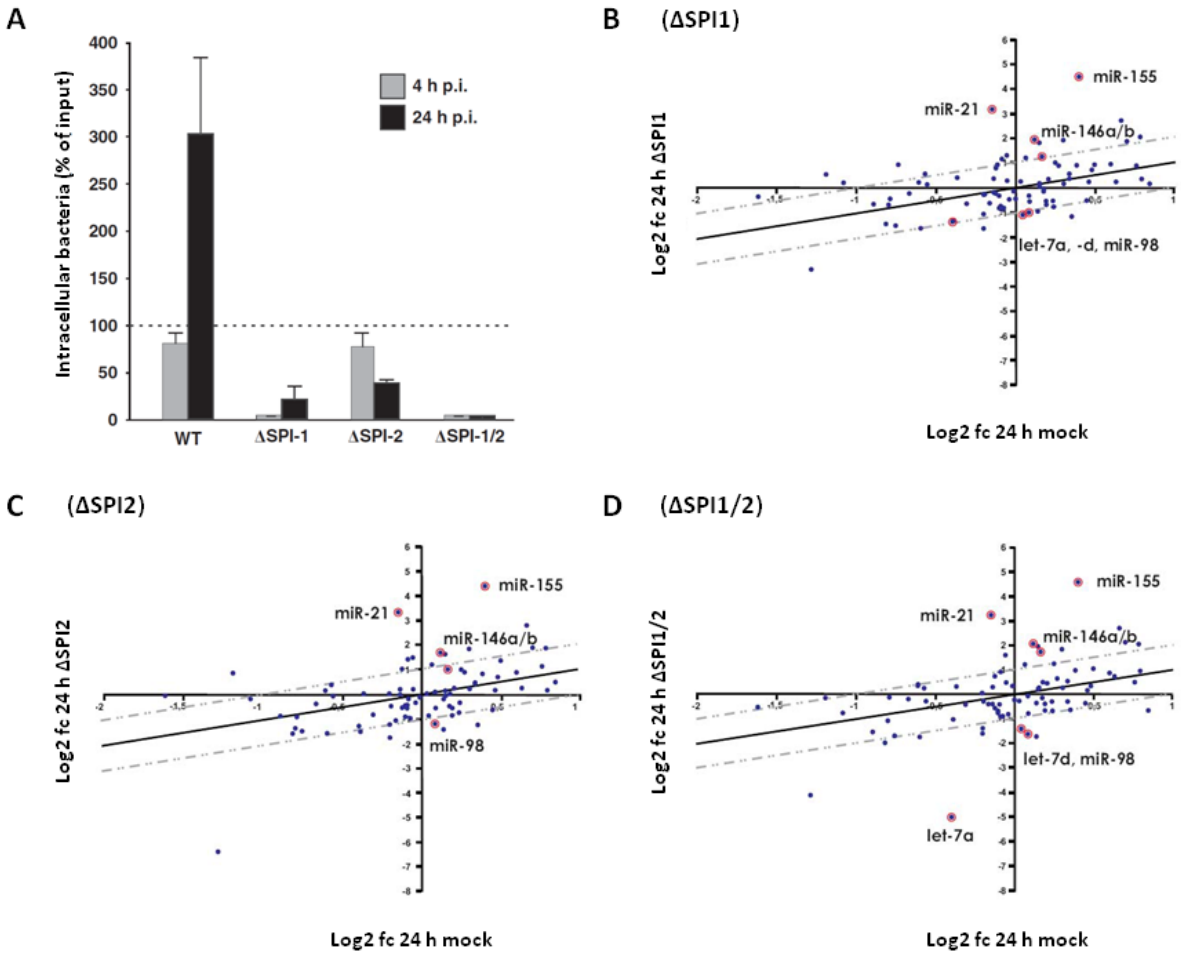

Figure 8: *S.* Typhimurium virulence mutants trigger similar miRNA expression changes as the wild-type strain in RAW264.7 macrophages. (A) Intracellular wild-type and attenuated (SPI-mutant) *S.* Typhimurium CFUs recovered from RAW264.7 macrophages at 4 and 24 h pi (presented as % of bacteria added to the cell culture supernatant at the onset of infection). **(B-D)** Log2 miRNA fold-changes comparing 0 h to 24 h control treatment (x-axis) and 0 h control treatment to 24 h *S.* Typhimurium challenge (y-axis), using either a SPI1 deletion strain (B), a SPI2 deletion strain (C) or a strain lacking both SPIs (D). The continuous line indicates a linear correlation between infection and control-treatment; dashed lines represent 2-fold up- and down-regulation cut-offs comparing infection and control-treatment.

High-throughput sequencing analysis of miRNA expression in RAW264.7 macrophages challenged with the three mutant *S.* Typhimurium strains demonstrated that regulation of miRNAs miR-21, miR-146, miR-155 and let-7 proceeded similarly to cells infected with the wild-type strain (Fig. 8B-D compared to Fig. 5A). This was validated experimentally by Northern blot (Fig. 9A) and by real-time PCR expression analyses of miR-146a, miR-155 and let-7a (Fig. 9B-D). The results suggest that miRNA expression changes upon challenge of macrophages with *S.* Typhimurium occur irrespective of

whether cells are actually infected (wild-type invasive *S.* Typhimurium) or have received an extracellular microbial stimulus only (i.e. ΔSPI1/2 mutant *S.* Typhimurium).

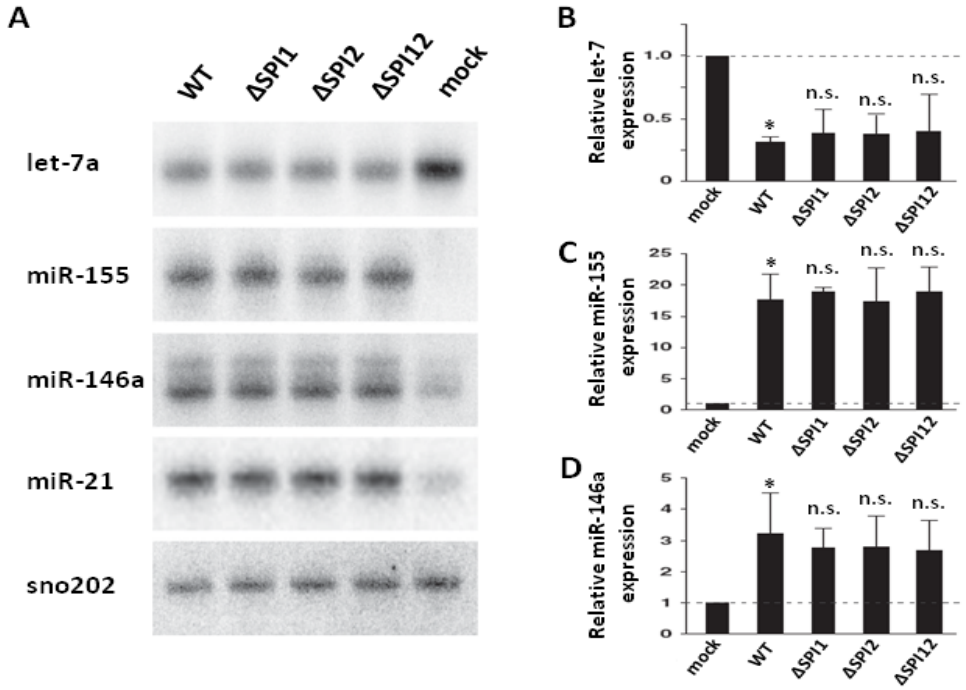

Figure 9: Validation of miRNA expression changes in *S.* Typhimurium infected RAW264.7 macrophages. (A) Northern blot analysis of miRNA expression 24 h post infection with wild-type *S.* Typhimurium, the indicated SPI-deletion mutants or control-treatment (mock). SnoRNA-202 served as a loading control. **(B-D)** Real-time PCR analysis of expression changes of let-7a (B), miR-155 (C) and miR-146a (D) 24 h post infection with wild-type *S.* Typhimurium or the indicated SPI-deletion mutants compared to control-treatment (mock). SnoRNA-202 served as a reference control. * indicates a significant difference in miRNA regulation compared to mock-treatment (*P*-value < 0.05); n.s. (not significant) denotes the lack of significant differences in miRNA regulation relative to WT-infection (P-value > 0.05).

To further test the hypothesis that *S.* Typhimurium forgos manipulation of the macrophage miRNA response, RAW264.7 cells were infected with a modified wild-type strain that expresses green fluorescent protein (GFP) from a genomic locus (Papenfort, et al., 2009). Macrophages challenged with GFP expressing *S.* Typhimurium were subjected to cell-sorting at 24 h pi in order to separate the cells that actually contained the bacteria from those that had possibly faced but not taken up the pathogen. Expression levels of miR-155, miR-146 and let-7a in the GFP-positive and the GFP-negative fraction of cells from the same sample were analyzed by real-time PCR. Expression was compared to a non-infected mock-treated control, which was sorted using the same settings as for the GFP-negative fraction of the infected sample (Fig. 10).

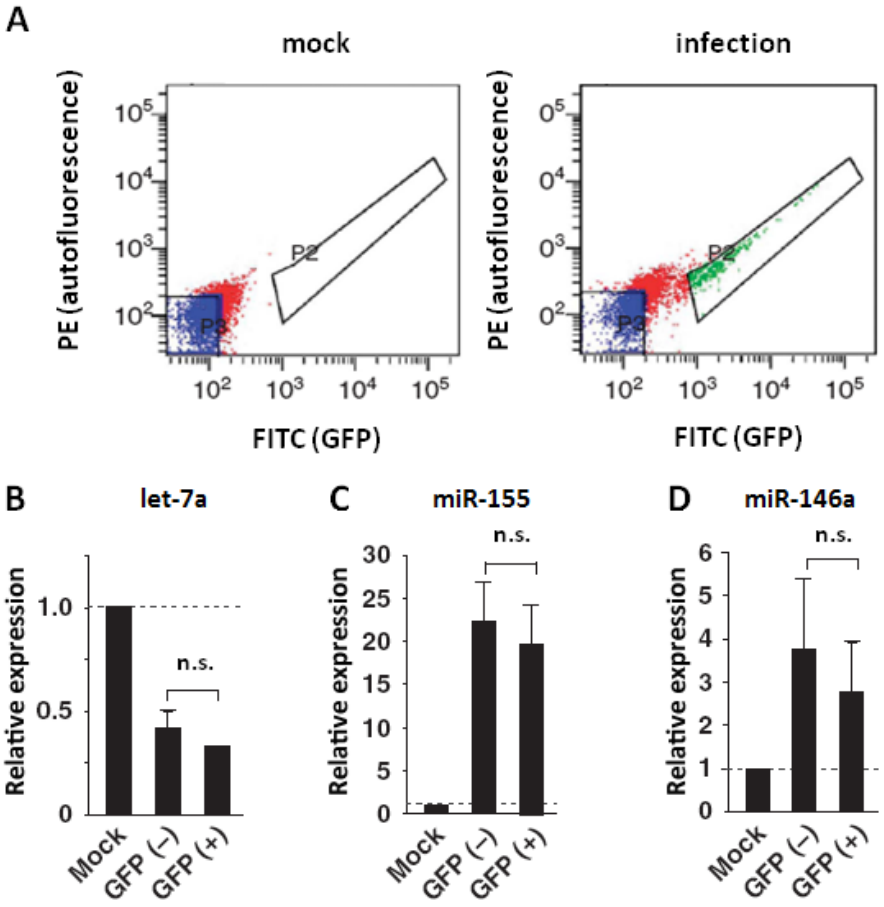

Figure 10: MiRNA regulation in *S.* Typhimurium challenged macrophages proceeds similarly in invaded and non-invaded cells. (A) Representative dot plots of auto- versus GFP-fluorescence in a non-infected and in a GFP-*Salmonella* infected culture of RAW264.7 macrophages. **(B-C)** Real-time PCR analysis of miRNA expression in 24 h *S.* Typhimurium-infected samples sorted for GFP-positive and GFP-negative (autofluorescent) cells compared to 24 h control-treated (non-infected) samples, sorted for auto-fluorescence. n.s. (not significant) denotes the lack of a significant difference in miRNA regulation (*P*-value > 0.05), comparing the indicated conditions

Flow-cytometric analysis showed that at an MOI of 1, used throughout this study, ~10 % of all cells of *S. Typhimurium* treated samples were actually infected 24 h pi (Fig. 10A). In the GFP-positive and the GFP-negative fraction of cells from the infection sample miR-146 and miR-155 were induced and let-7a was decreased with no significant difference between the two populations, confirming that the extracellular stimulus rather than the actual infection triggers the observed miRNA expression changes (Fig. 10B-D).

As the miRNA response to *S.* Typhimurium proceeds similarly in host cells that are actually invaded or have received an extracellular microbial stimulus only, it was hypothesized that even purified *S.* Typhimurium agonists of host plasmamembrane PRRs can trigger the observed miRNA regulations. Therefore, macrophages were

challenged with purfied *S*. Typhimurium cell-wall component LPS or flagellum protein FliC, which activate the major plasmamembrane PRRs TLR4 and TLR5 respectively. Challenge of macrophages with purified *S*. Typhimurium LPS induced miR-155 and reduced let-7 expression similar to wild-type *S*. Typhimurium infection (Fig. 11A, B). FliC on the other hand did not trigger regulation of these miRNAs (Fig. 11A, B), consistent with its receptor TLR5 hardly being expressed by RAW264.7 macrophages (Mizel, et al., 2003).

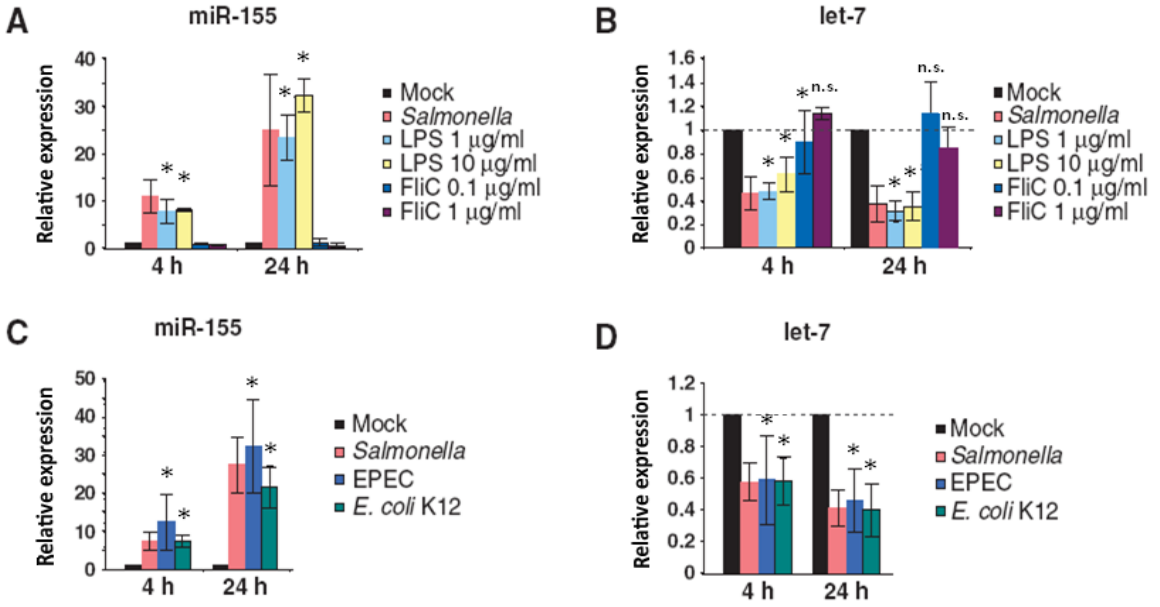

Figure 11: miRNA regulations upon *S*. Typhimurium challenge, reproduced with pure LPS or pathogenic / non-pathogenic *Escherichia coli*. (A-B) Real-time PCR analysis of miR-155 and let-7a expression 4 and 24 hours post *S.* Typhimurium infection or treatment with LPS or FliC (doses indicated), compared to mock-treatment. **(C-D)** Real-time PCR analysis of miR-155 and let-7a expression upon challenge with *S.* Typhimurium, enteropathogenic *E. coli* (EPEC) or *E. coli* K12 for 24 h. * denotes significant regulation compared to mock-treatment (*P*-value < 0.05), while n.s. (not significant) denotes lack of significance (*P*-value > 0.05).

If LPS triggers the host miRNA response to *S*. Typhimurium, other bacteria (pathogenic or non-pathogenic) that expose LPS should trigger the same miRNA regulations. Indeed, enteropathogenic *Escherichia coli* (EPEC) or non-pathogenic *Escherichia coli* (*E. coli* K12) induced miR-155 and reduced let-7 expression similar to *S*. Typhimurium infection in macrophages (Fig. 11C and D).

To prove that *S*. Typhimurium LPS triggers the here discovered down-modulation of let-7 in macrophages, bone-marrow derived macrophages (BMDMs) from wild-type and TLR4$^{-/-}$ C57BL/6 mice were incubated with heat-killed *S*. Typhimurium (HKS);

challenge with live *S*. Typhimurium would rapidly trigger programmed cell-death in BMDMs (Miao, et al., 2010_a). Let-7 expression was monitored for several days in order to get an impression of the time-frame of let-7 down-regulation during the anti-microbial response. In BMDMs from wild-type but not from TLR4$^{-/-}$ animals let-7 expression was continuously reduced in response to HKS-stimulation, even proceeding until day 5 post onset of HKS stimulation (Fig. 12). Yet, let-7 regulation in BMDMs was delayed compared to RAW264.7 cells. In summary these results suggest that the newly identified down-regulation of let-7 miRNAs in macrophages in response to microbial challenge is triggered by TLR4 sensing of bacterial LPS.

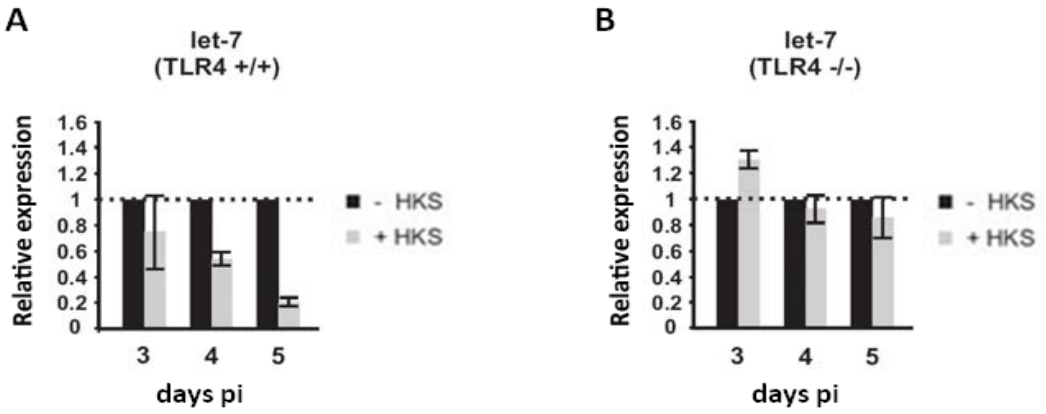

Figure 12: let-7a down-regulation in *S*. Typhimurium-challenged bone marrow-derived macrophages (BMDMs) depends on TLR4. Expression of let-7a was monitored by real-time PCR on days 3, 4 and 5 post HKS stimulation of TLR4+/+ (wild-type) murine BMDMs **(A)** or TLR4-/- murine BMDMs **(B)**. Expression of let-7a in HKS stimulated cells is presented as relative expression compared to control-treated BMDMs, respectively (- HKS).

6.3 Functions of let-7 in the macrophage response to *S. Typhimurium*

While miR-146 and miR-155 regulate the expression of key cellular signal transduction components downstream of LPS sensing (section 4.3.2), the role of let-7 in the macrophage inflammatory response remained to be determined. Bio-computational predictions by the Targetscan algorithm (targetscan.org) list the messengers of major macrophage cytokines IL6 and IL10 as putative targets of let-7 miRNAs. As depicted in Fig. 13A, seed-complementary sequences to miRNA let-7a in the 3'UTRs of IL6 and IL10 mRNA were also detected by the RNAhybrid algorithm. Alignment of the IL6 or the IL10 3'UTR sequences of several mammalian species shows that the predicted let-7 target sites are evolutionarily conserved (Fig. 13B).

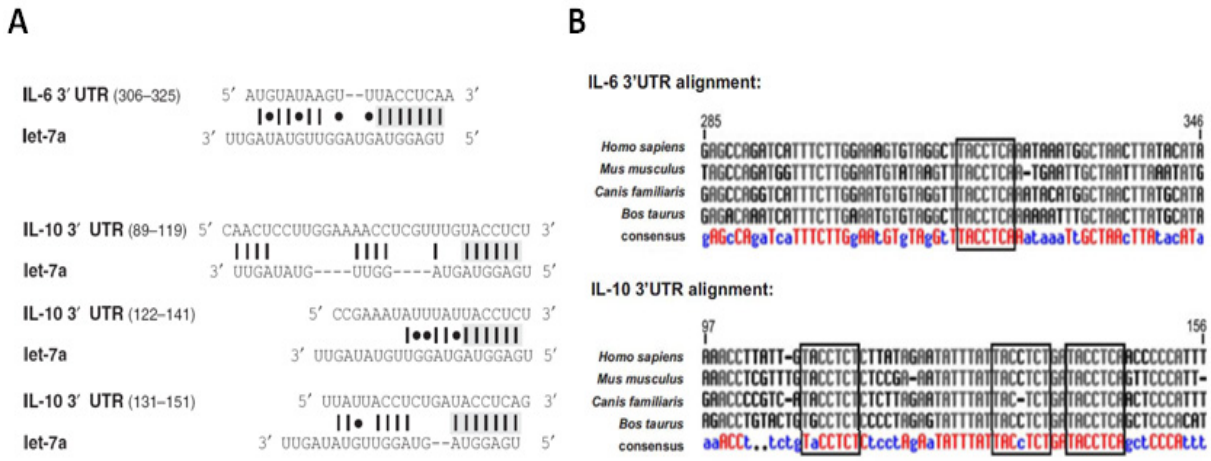

Figure 13: Computational analysis predicts let-7 binding-sites within the 3'UTRs of IL6 and IL10 mRNAs. **(A)** RNAhybrid-prediction of let-7a binding sites within the murine IL6 and IL10 mRNA sequences at the indicated 3'UTR nucleotide positions. Lines indicate Watson-Crick base pairings, dots denote wobble base pairs. Predicted miRNA seed-pairing sites are highlighted in grey. **(B)** Sections of alignments of the IL6 or IL10 3'UTR sequences of the indicated vertebrate species. Black squares indicate the predicted let-7 seed-pairing sites. Alignments were performed using the Multalin algorithm (Corpet, 1988). 3'UTR equences were extracted from the ENSEMBL genome browser.

While IL6 is a major pro-inflammatory cytokine, IL10 rather functions to limit the pathological effects of IL6 and other inflammation mediators (Couper, et al., 2008). To determine whether let-7 controls expression of these key macrophage cytokines the 3'UTR sequences of the IL6 and IL10 mRNAs were cloned downstream of the *Renilla* luciferase ORF of the pSICHECK-2™ dual luciferase reporter plasmid. A second luciferase (Firelfy luciferase) expressed from the same plasmid serves as a normalization control. The luciferase-3'UTR reporters of IL6 and IL10 were transfected into mouse embryonic fibroblast (MEF) cells and luciferase activites in response to delivery of synthetic let-7 miRNA mimics was determined. Other than macrophages, MEF cells do not regulate let-7 upon *S.* Typhimurium infection, thus providing a neutral system (Fig 14D). As shown in Fig. 14A and B, neither control nor let-7a and let-7d miRNA mimics affected the activity of *Renilla* luciferase expressed from the empty reporter plasmid (not harbouring the IL6 or IL10 3'UTR). When MEF cells were however transfected with the plasmids harboring either the IL6 or the IL10 3'UTR downstream of the *Renilla* ORF let-7 mimic co-transfection markedly reduced *Renilla* luciferase activity (Fig. 14A and B). Co-transfection of a control miRNA mimic, on the other hand, did not affect reporter activity (Fig. 14A and B).

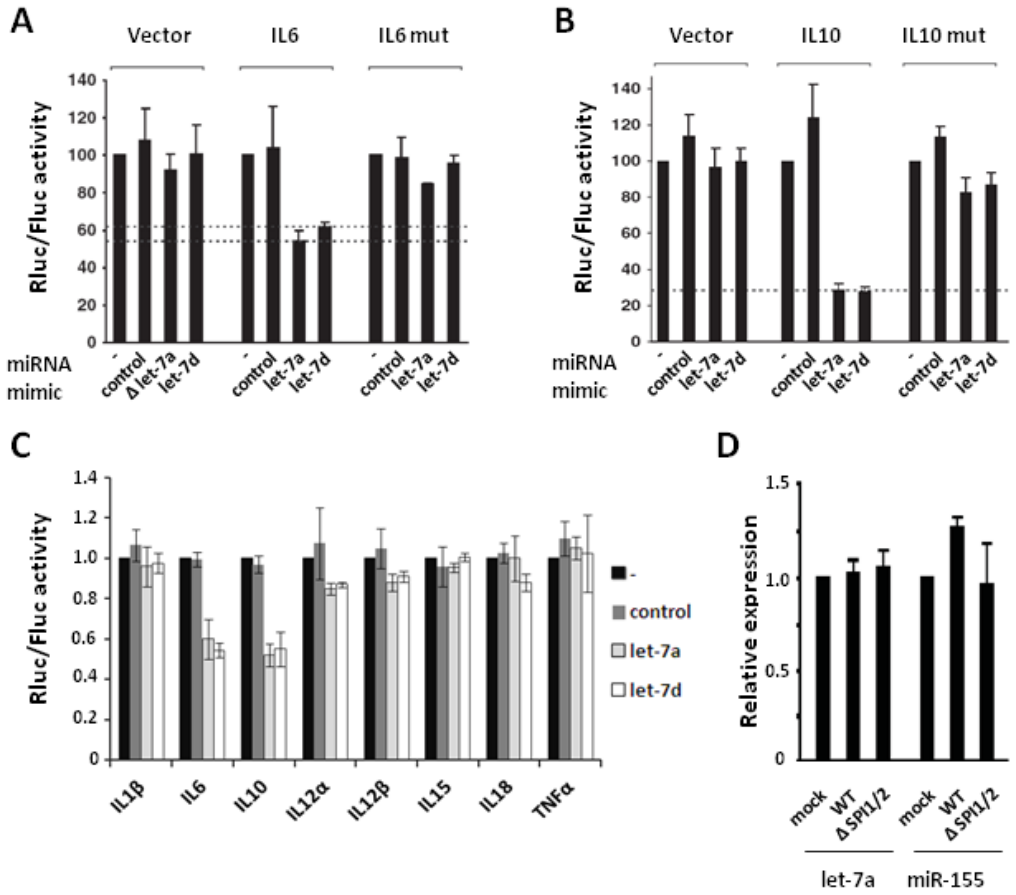

Figure 14: let-7 targets IL6 and IL10. (A) Regulation of a reporter construct harboring the murine IL6 3'UTR sequence down-stream of a *Renilla* luciferase (Rluc) ORF compared to a co-expressed normalization luciferase (Fluc). "Vector" refers to the empty reporter plasmid, "IL6" to the plasmid harbouring the IL6 3'UTR and "IL6mut" to the reporter with the predicted IL6 3'UTR target site of let-7 mutated. The synthetic miRNA mimics co-expressed with the respective plasmids are indicated at the bottom of the figure. **(B)** Same as in (A) but with the murine IL10 3'UTR sequence fused to the *Renilla* ORF; all three predicted let-7 binding sites are mutated in the IL10mut construct. **(C)** Analysis of the repression of 3'UTR luciferase reporters of prominent macrophage cytokines by let-7. **(D)** Real-time PCR analysis of let-7a and miR-155 expression in MEF-cells challenged with the indicated *S*. Typhimurium strains compared to mock-treatment.

When the whole let-7 seed-complementary site of the IL6 reporter (Fig. 13A) was mutated (scrambled, see table 4, section 8.11), regulation in response to the let-7 mimics was abrogated (Fig. 14A). Upon scrambling of all three predicted let-7 seed-complementary sites within the IL10 reporter sequence (Fig. 13A) regulation was lost as well (Fig. 14B). Importantly, targeting of cytokine mRNAs by let-7 seemed to be specific to IL6 and IL10 as none of the other tested 3'UTR reporters of prominent macrophage cytokines was suppressed by let-7 (Fig. 14C).

After let-7 over-expression had been shown to repress IL6 and IL10 3' UTR reporter activity (Fig. 14), it was investigated whether down-regulation of the let-7 miRNA family in microbially challenged macrophages (Fig. 5) in turn may elevate the activities of the reporters. To this end the IL6 and IL10 3' UTR reporters with intact or mutated let-7 binding sites were transfected into RAW264.7 macrophages. Upon stimulation with *S*. Typhimurium (to trigger down-regulation of let-7) IL6 and IL10 reporter activity was elevated (Fig. 15). Upon let-7 binding-site mutation however, regulation was lost in both cases (Fig. 15). As a control the experiment was repeated in MEF cells, which do not regulate let-7 in response to *S*. Typhimurium (Fig. 14D). As expected no regulation of the cytokine reporters was observed in MEF cells (Fig. 15).

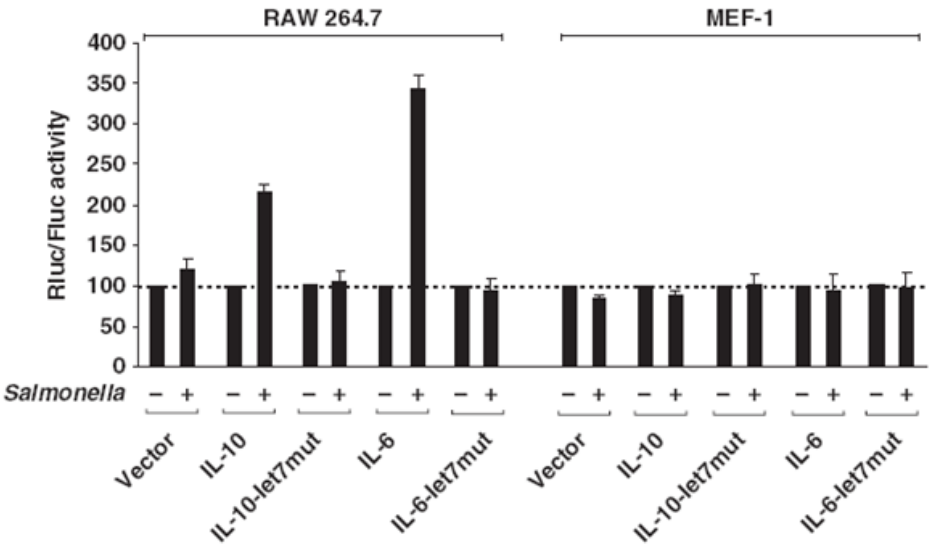

Figure 15: *S.* Typhimurium challenge elevates IL6 and IL10 reporter activity in RAW264.7 macrophages. *Renilla* luciferase (Rluc) reporters described in Fig. 14 were analysed for their regulation relative to the Firefly normalization luciferase (Fluc) upon 24 h *S.* Typhimurium infection compared to mock-treatment.

To determine whether let-7, besides the IL6 and IL10 reporter activities, affects actual cytokine production RAW264.7 macrophages were transfected with rising doses of let-7 mimics. IL6 and IL10 protein production was determined by ELISA. The effect of let-7 mimic transfection on IL6 and IL10 3'UTR reporter activity was monitored in parallel to assess whether any observed effect on IL6 and IL10 production may be explained by 3'UTR mediated repression. Transfection of macrophages with rising doses of let-7a or let-7d mimics (2.5 – 40 nM) gradually reduced both IL6 and IL10 reporter activity (Fig. 16A, C) and cytokine production (Fig. 16B, D).

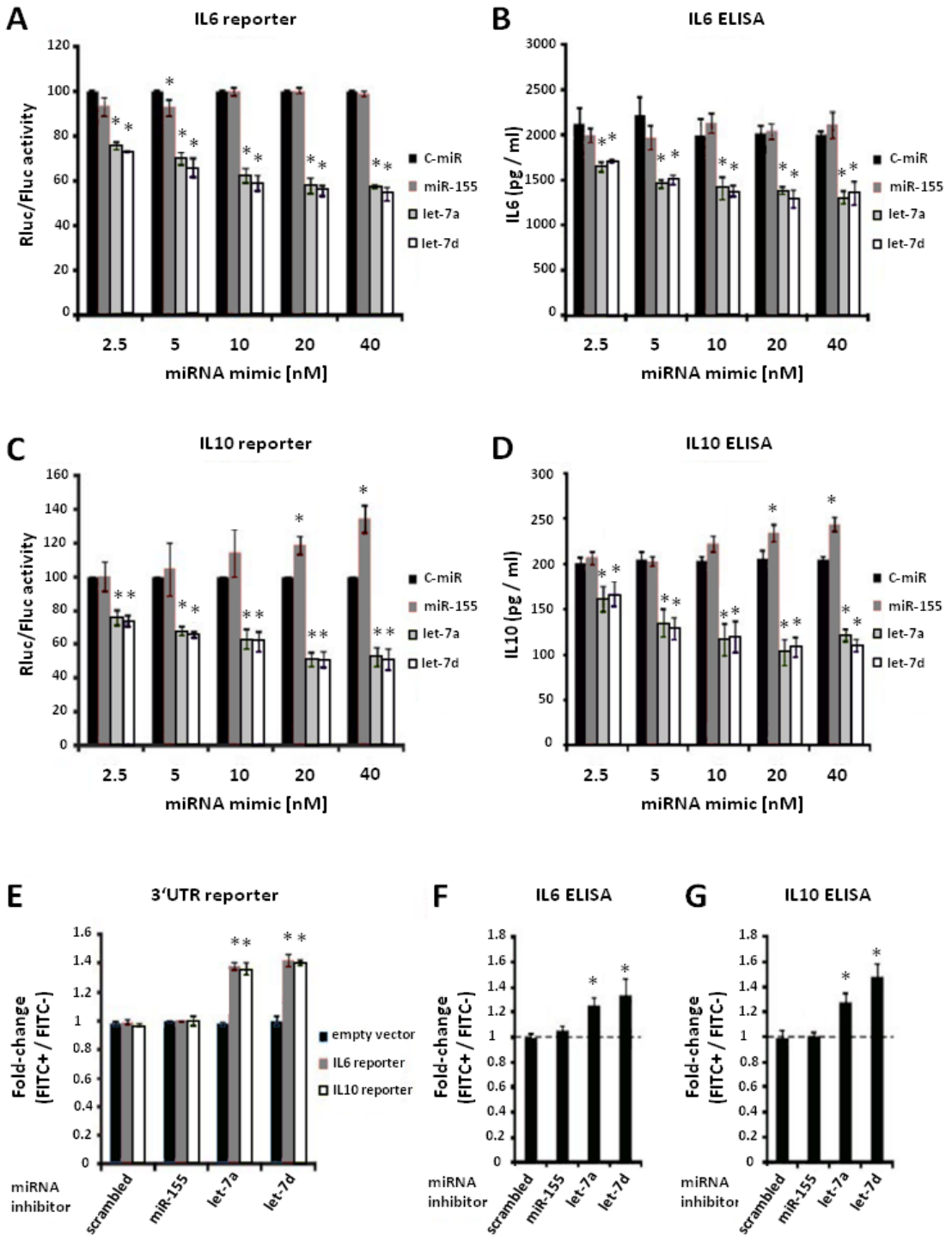

Figure 16: let-7 miRNA modulation in RAW264.7 macrophages impacts IL6 and IL10 production. **(A, C)** Regulation of IL6 and IL10 reporters (introduced in Fig. 14) upon 24 h of HKS stimulation and miRNA mimic delicery. **(B, D)** Same experimental setup as in (A) and (C) but IL6 and IL10 protein production measured (ELISA) instead of using luciferase reporters. **(E)** IL6-, IL10- and control-reporter activity measured upon transfection of cells with FITC-labeled miRNA inhibitors and cell-sorting for the FITC-positive (inhibitor containing) and FITC-negative populations. **(F-G)** Same experimental setup as in (E) but cytokine IL6 (F) and IL10 (G) production measured by ELISA instead of using luciferase reporters. Asterisks indicate regulations significantly different from the respective controls (*P*-value < 0.05).

To assess whether inhibition of endogenous let-7 in macrophages in turn elevates IL6 and IL10 production, RAW264.7 cells were transfected with 3' FITC conjugated LNA™ miRNA antisense-inhibitors. Cells were enriched for the population that had taken up the inhibitor by cell sorting based on the FITC fluorophore. The FITC-negative fraction was collected as well and IL6 and IL10 reporter activity and protein production were determined in both fractions. Upon transfection of a control (scrambled) miRNA inhibitor reporter activity and cytokine production in the FITC-positive fraction (harboring the inhibitor) was comparable to the FITC-negative fraction (not harboring the inhibitor). However, let-7a or let-7d inhibitor transfection significantly increased reporter activity (Fig. 16E), and IL6 (Fig. 16F) or IL10 (Fig. 16G) production in the FITC-positive populations compared to the FITC-negative populations of cells. In summary, these results identify down-regulation of let-7 under conditions of microbial challenge as a novel layer in the regulation of cytokine expression in macrophages via relieve of post-transcriptional repression of IL6 and IL10.

6.4 Analysis of *S. Typhimurium* responsive microRNAs in LPS tolerant cells

The above described experiments address the role of miRNA in the primary response of macrophages to microbial challenge. Yet, macrophages, upon pre-exposure to microbial PAMPs such as LPS, may enter a state of LPS hypo-responsiveness that is referred to as endotoxin-tolerance and likely protects from inflammation induced pathologies (Biswas and Lopez-Collazo, 2009). Currently, the cellular pathways involved in the maintenance of endotoxin-tolerance as well as the ability of endotoxin-tolerant host-cells to respond to pathogens remain poorly defined. The observation that miRNA expression changes in response to *S.* Typhimurium are largely triggered by its LPS (Fig. 11A, B; Fig. 12) suggests that in endotoxin-tolerant macrophages the miRNA response to the pathogen is impared. To address this, RAW264.7 macrophages were pre-exposed to HKS for 24 h and then incubated in the absence of a microbial stimulus for another 5 days, allowing for decay of the initial inflammatory response. A second stimulus was then provided by challenge with wild-type *S.* Typhimurium or the three previously introduced virulence mutants (ΔSPI1, ΔSPI2, ΔSPI1/2). Endotoxin tolerance induction was verified by microarray analysis of NFκB dependent gene expression changes in response to wild-type or ΔSPI1/2 *S.* Typhimurium mutant stimulation (Fig. 17).

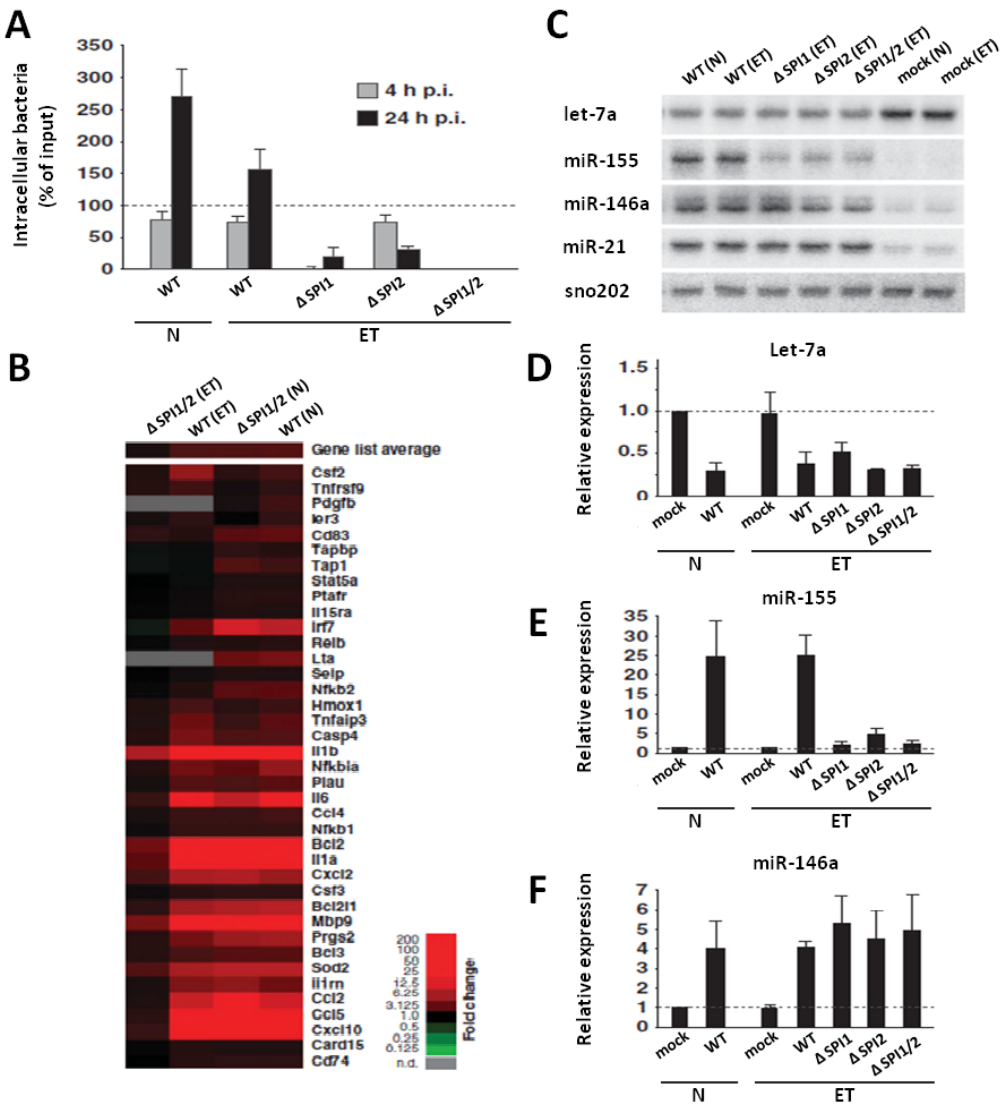

Figure 17: Suppression of the inflammatory response to microbes impacts miRNA regulation in endotoxin-tolerant macrophages (A) Invasion (4 h pi) and intracellular replication (24 h pi) of *S.* Typhimurium in naive and endotoxin-tolerant (ET) cells. **(B)** Microarray analysis of the induction of NFκB responsive genes upon challenge of naïve and ET cells with wild-type or ΔSPI1/2 *S.* Typhimurium. **(C)** Northern blot analysis of miRNA expression upon challenge of naive and ET macrophages with wild-type *S.* Typhimurium or the indicated SPI mutants. **(D-F)** Same experiment as in (C) but quantifying miRNA expression changes by real-time PCR.

Host-cell invasion and intracellular replication of the four *S.* Typhimurium strains in endotoxin-tolerant macrophage cultures proceeded similar to naïve macrophages, as determined by CFU recovery assays; yet, a slight drop in replication was observed for the wild-type strain (Fig. 17A). Microarray analysis confirmed the activation of NFκB dependent genes upon challenge of naive cells with wild-type *S.* Typhimurium or the ΔSPI1/2 strain 24 h post infection (Fig. 17B). By contrast, in endotoxin-tolerant

RAW264.7 macrophages stimulated with the strictly extracellular ΔSPI1/2 strain NFκB dependent gene expression was largely suppressed (Fig. 17B). Strikingly, infection of endotoxin-tolerant macrophages with wild-type *S.* Typhimurium restored NFκB dependent gene expression as compared to naïve macrophages (Fig. 17B). Irrespective of the absence of an inflammatory response, let-7, miR-146 and miR-21 were regulated normally in endotoxin-tolerant cells challenged with the *S.* Typhimurium ΔSPI1/2 mutant (Fig. 17C, D, F). Also the *S.* Typhimurium ΔSPI1 and ΔSPI2 single mutants triggered let-7, miR-146 and miR-21 regualtion (Fig. 17C, D, F). On the other hand miR-155 behaved rather like the NFκB dependent mRNAs (see Fig. 17B): it stayed largely mute in endotoxin-tolerant macrophages challenged with the virulence mutants but was reactivated along with general NFκB dependent gene expression by wild-type *S.* Typhimurium (Fig. 17C, E). These results demonstrate miR-146 and the cytokine regulator let-7 to function in the first line of macrophage defense, since their down-regulation upon microbial stimulation proceeds even in the absence of an inflammatory response. These observations also show that the previously observed co-induction of miR-146 and miR-155 in macrophages (Quinn and O'Neill, 2011) is not maintained under conditions of attenuated NFκB dependent transcription. This predicts distinct functions of both miRNAs at sub- and pro-inflammatory macrophage activity, respectively, which was examined further.

6.5 Thresholds of miR-146 and miR-155 regulation in the LPS response

As delineated above miR-155, but not miR-146, remains suppressed in endotoxin-tolerant macrophages challenged with attenuated *S.* Typhimurium (Fig. 17C-F). This is surprising, given that both miRNAs were shown to be induced by the pro-inflammatory transcription factor NFκB (Quinn and O'Neill, 2011). LPS is responsible for major *S.* Typhimurium-induced host miRNA expression changes (Fig. 11A, B; Fig. 12). Therefore, the uncoupling of miR-146 and miR-155 expression in endotoxin-tolerant macrophages might indicate a higher sensitivity of miR-146 to LPS nduced NFkB activity as compared to miR-155, allowing miR-146 to be activated even by the minute residual activity of the LPS sensor TLR4 (Fig. 17C, F). To test this hypothesis of distinct sensitivities to TLR4 activity, RAW264.7 macrophages were challenged with rising doses of *S.* Typhimurium LPS (0.01-1000 ng / ml) and expression of miR-146a, miR-146b and miR-155 was

monitored by real-time PCR both at the level of the mature processed miRNAs and the primary transcripts (pri-miRNAs).

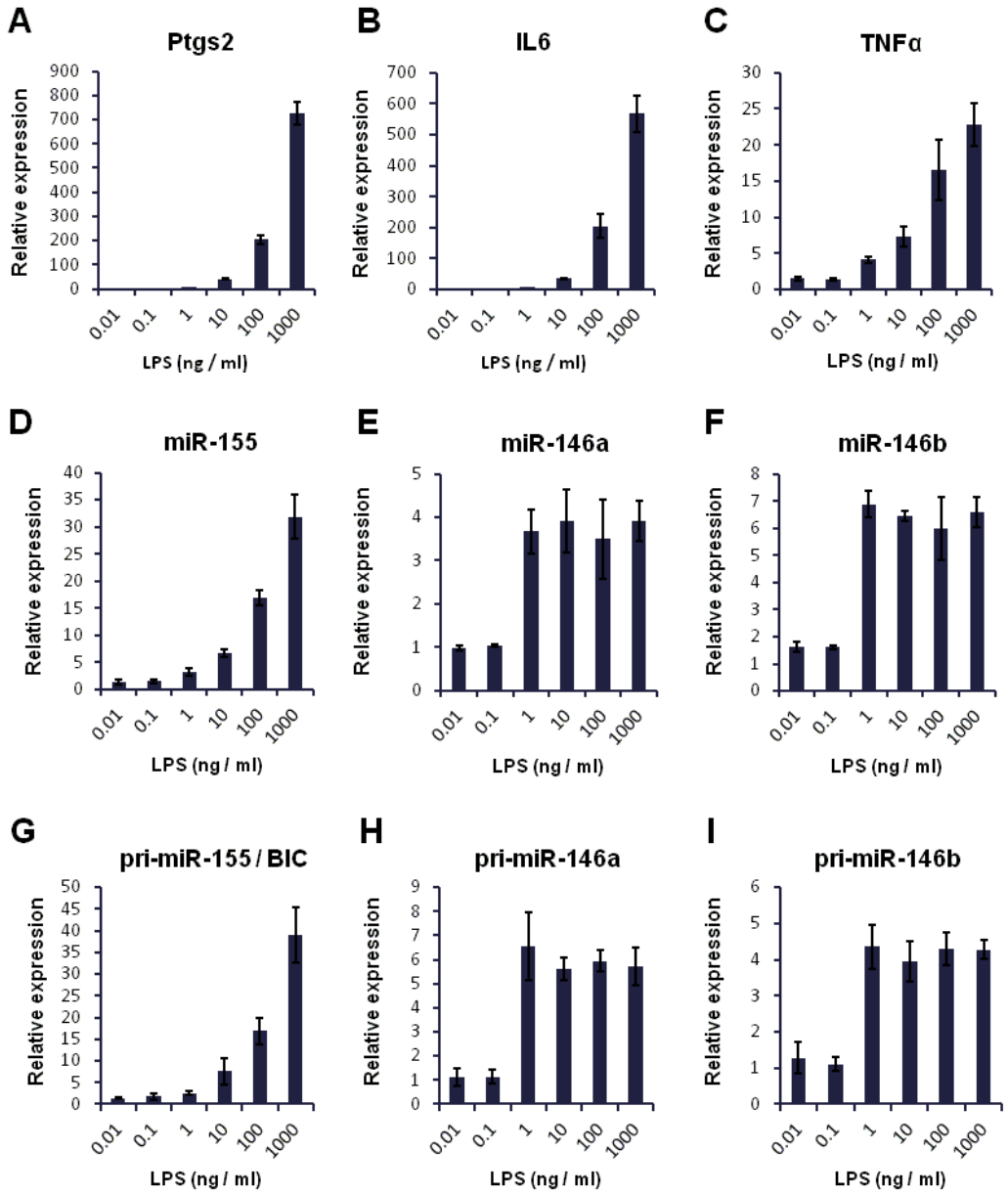

Figure 18: Different LPS activation thresholds of miR-155 and miR-146 in RAW264.7 macrophages. **(A-C)** Expression changes of the mRNAs of inflammation markers Ptgs2, IL6 and TNFα were determined by real-time PCR upon treatment with rising doses of LPS (0.01-1000 ng / ml) for 24 h, relative to control-treatment (no LPS). **(D-F)** Same experiment as in (A-C) but monitoring the expression of mature miR-155, miR-146a and miR-146b. **(G-I)** Same experiment as in (A-C) but monitoring the expression of the primary transcripts of miR-155, miR-146a and miR-146b.

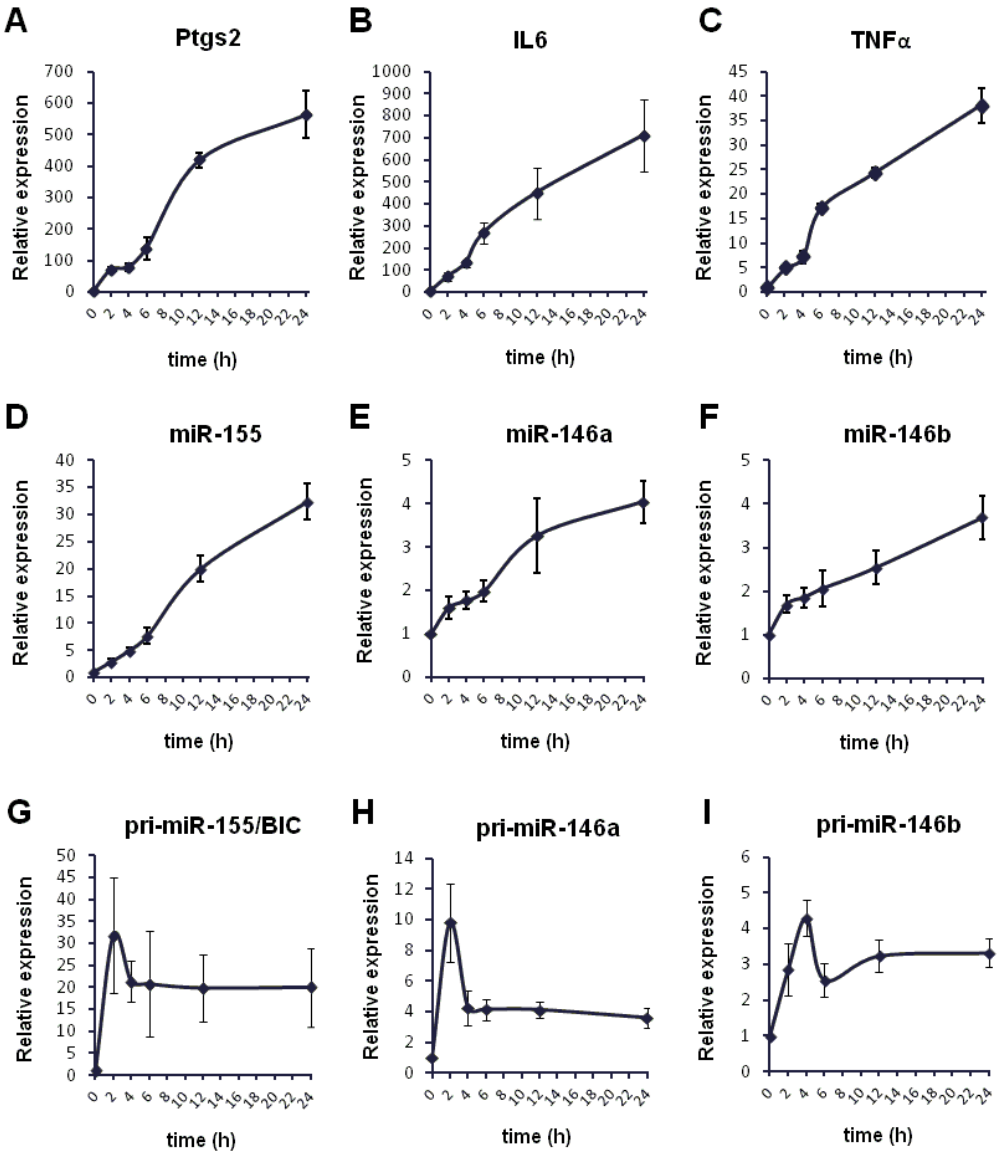

Figure 19: Similar kinetics of miR-155 and miR-146 induction in LPS-challenged RAW264.7 macrophages. (A-C) Expression changes of the mRNAs of inflammation markers Ptgs2, IL6 and TNFα, determined by real-time PCR upon treatment with 1000 ng LPS / ml for 24 h, relative to control-treatment (no LPS). **(D-E)** Same experiment as in (A-C) but monitoring the expression of mature miR-155, miR-146a and miR-146b. **(G-I)** Same experiment as in (A-C) but monitoring expression of the primary transcripts of miR-155, miR-146a and miR-146b.

As positive controls for LPS-induced inflammatory gene expression the messengers of established macrophage inflammation markers Ptgs2 (prostaglandin E2 synthase), IL6 and TNFα were monitored by real-time PCR. As expected (Kalis, et al., 2003), the inflammation marker mRNAs were gradually induced in response to rising doses of LPS (Fig. 18A-C), reaching highest expression at 1000 ng LPS / ml. Expression of miR-155 mature and primary transcript (the latter is also known as BIC non-coding RNA)

exhibited a similar LPS-dose dependence as the inflammation markers (Fig. 18D, G), in line with the finding that miR-155 is tightly coupled to the general macrophage inflammatory response (Fig. 17). In sharp contrast, mature and primary miR-146a and miR-146b transcript expression was switched on at a low dose of LPS (1 ng / ml) that did not trigger relevant expression of inflammation markers Ptgs2, IL6 or TNFα (Fig. 18E, F, H, I). Irrespective of the different LPS sensitivities, the kinetics of mature miR-146a, miR-146b and miR-155 induction, monitored over 24 h at a fixed high concentration of LPS (1000 ng / ml), were largely comparable and followed the kinetics of inflammation markers Ptgs2, IL6 and TNFα (Fig. 19). Overall these results suggest that regardless of similar induction kinetics in response to a high dose of LPS, miR-146 and miR-155 are activated at different thresholds of TLR4 activity. That is, miR-146 is saturatingly induced at sub-inflammatory doses of LPS, while miR-155 is coupled to the induction of inflammatory marker genes, triggered at higher doses of LPS.

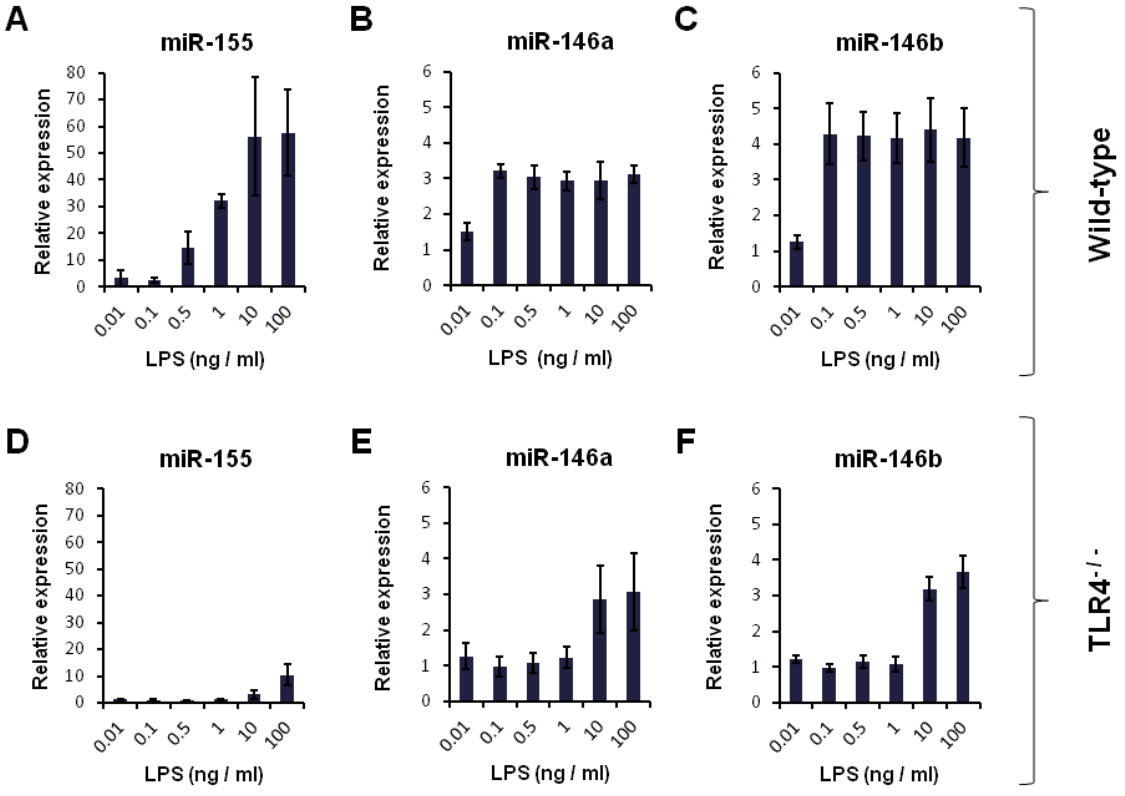

Figure 20: TLR4 mediates differential activation of miR-155 and miR-146 in LPS challenged BMDMs. (A-C) Real-time PCR analysis of mature miR-155 (A), miR-146a (B) and miR-146b (C) expression in wild-type BMDMs stimulated with rising doses of LPS (0.01-1000 ng / ml) for 8 h. **(D-E)** Same experiment as in (A-C) but using homozygous TLR4 knockout (TLR4$^{-/-}$) BMDMs.

To validate that the TLR4 receptor mediates differential activation of miR-146 and miR-155 upon challenge of macrophages with rising doses of *S.* Typhimurium LPS, miRNA expression was monitored in BMDMs from wild-type and TLR4 deficient mice. In wild-type BMDMs miR-146a and miR-146b expression was switched on saturatingly at 0.1 ng LPS / ml (~3-fold and ~4-fold, respectively, Fig. 20B, C), while miR-155 was gradually induced, reaching saturation at 10 ng LPS / ml (~60-fold, Fig. 20A). In BMDMs lacking both alleles of the TLR4 gene switch-induction of miR-146a and miR-146b required a 100-times higher concentration of LPS (10 ng LPS / ml, Fig. 20E, F) compared to wild-type cells; miR-155 did not saturate over the tested range of LPS doses in TLR4 deficient macrophages. Yet, mild induction of miR-155 was observed at 10 and 100 ng LPS / ml (~3- and ~10-fold, respectively, Fig. 20D). This demonstrates that the differential activation of miR-146 and miR-155 in LPS-stimulated wild-type macrophages is indeed mediated by TLR4 in the relevant window of concentrations (0.01 – 10 ng LPS / ml).

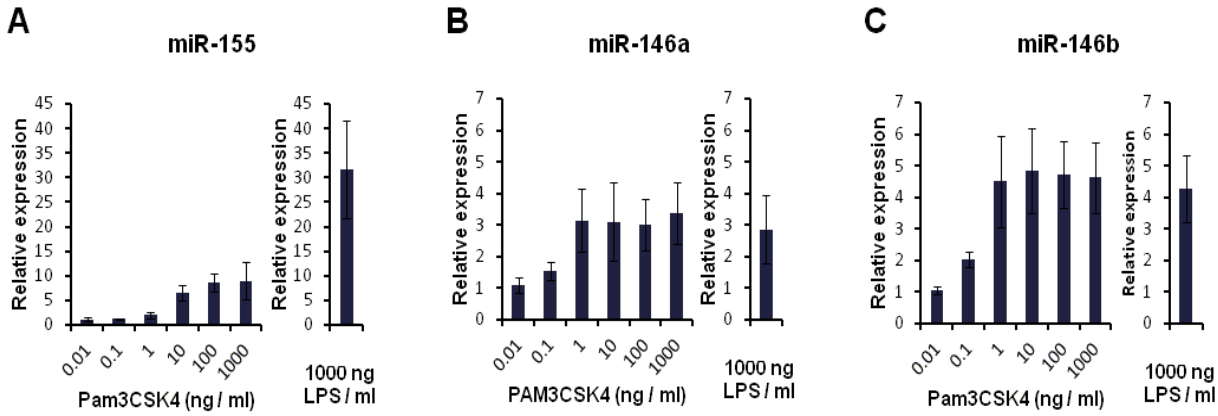

Figure 21: Different activation thresholds of miR-155 and miR-146 in RAW264.7 macrophages challenged with TLR2 ligand Pam3CSK4. (A) Real-time PCR analysis of mature miR-155 expression upon challenge with rising doses of Pam3CSK4 (0.01-1000 ng / ml) or 1000 ng LPS / ml for 24 h. **(B, C)** same experiment as in (A) but monitoring miR-146a (B) or miR-146b (C) expression.

Importantly, the differential PAMP sensitivity of miR-146 and miR-155 is not restricted to TLR4 sensing of LPS: RAW264.7 macrophages challenged with rising doses of Pam3CSK4, a microbial lipopeptide that activates macrophage surface receptor TLR2, triggered saturating induction of miR-146a and miR-146b at low doses of the PAMP (Fig. 21B, C), followed by miR-155 at higher doses (Fig. 21A). While Pam3CSK4 induced miR-146 to similar levels as did LPS (Fig. 21B, C), miR-155 induction by Pam3CSK4 saturated

at ~10-fold as compared to ~30-fold upon LPS treatment (Fig. 21A). This is in line with Pam3CSK4 being a weaker inflammation inducer than LPS (Chaurasia, et al., 2010; Ramsey, et al., 2008). This shows that saturating induction of miR-146 may also precede miR-155 induction when macrophages are stimulated with rising doses of microbial PAMPs other than LPS.

Collectively these results demonstrate that, despite similar induction-kinetics, miR-146 and miR-155 are activated at different thresholds of TLR activity. While miR-146 responds to minute *S.* Typhimurium LPS stimuli that do not trigger inflammation marker genes, miR-155 expression stays strictly coupled to the inflammatory response. Differential activation of miR-146 and miR-155 is not restricted to TLR4 but may also occur downstream of other TLRs.

6.6 Macrophage gene regulatory networks of miR-146 and miR-155

The differential association of miR-146 and miR-155 with macrophage inflammatory gene expression downstream of TLR stimulation suggested that these miRNAs may have different functions and target different mRNAs. To dissect their target-profiles, known and predicted targets of miR-146 and miR-155 in macrophage inflammatory signal transduction were tested for repression by both miRNAs. For miR-155 these were TAB2 (involved in TLR and cytokine receptor triggered NFκB activation) and IKKε (involved in anti-viral cytokine production downstream of TLRs), (Kawai and Akira, 2006), (Fig. 22A, B). A manual search for miR-155 seed-pairing sites within the 3'UTRs of genes associated with macrophage inflammatory signal transduction additionally revealed a 7-mer miR-155 seed-complementary site within the 3'UTR of NIK kinase (Fig. 22B). NIK has been implicated in NFκB activation downstream of TLR4 (Beutler, 2000), (Fig. 22A). Importantly, the miR-155 target site in NIK mRNA is deeply conserved among vertebrates (Fig. 22C). Therefore, NIK was included as a potential target of miR-155. Furthermore, the presently known miR-146 targets in the macrophage inflammatory response, TRAF6 and IRAK1, which constitute essential TLR4 signal transduction components (section 4.3.2, Fig. 22A), were tested for repression by both miR-146 and miR-155 (Fig. 25).

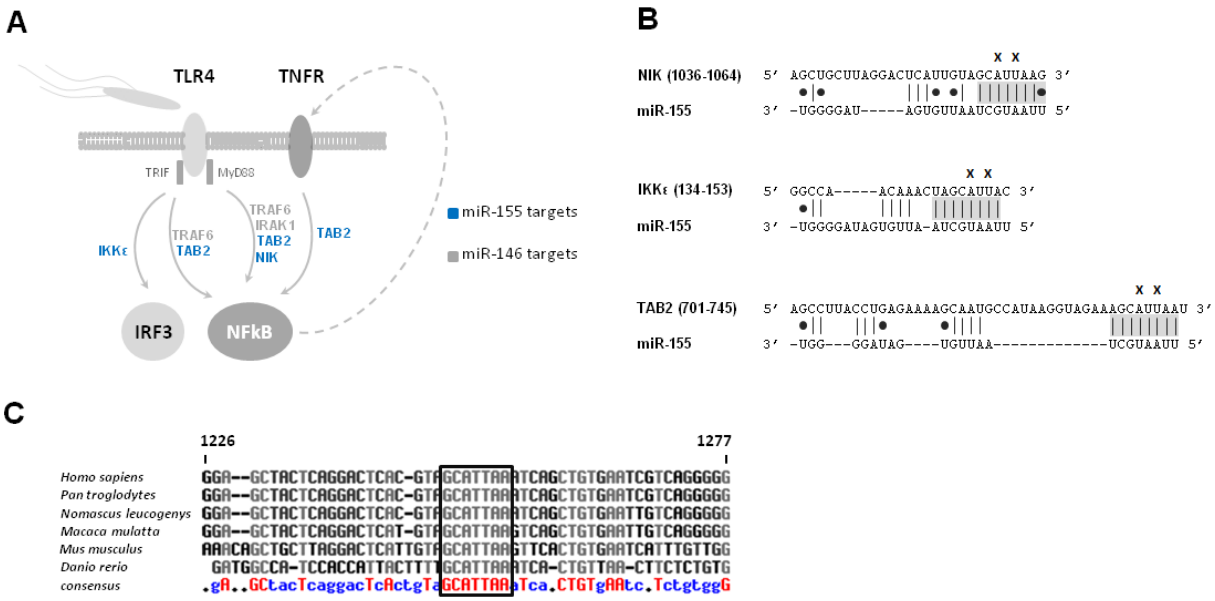

Figure 22: Known and putative miRNA targets in the macrophage TLR4 response. **(A)** Scheme of miR-146 and miR-155 targets in macrophage signaling pathways downstream of TLR4 activation (adapted from Kawai and Akira, 2006). Continuous arrow-lines indicate intracellular signaling pathways triggering pro-inflammatory transcription downstream of TLR4; dashed arrow-line indicates secondary autocrine/paracrine stimulation of pro-inflammatory transcription via TNFα release. **(B)** Binding sites of miR-155 within the 3'UTRs of the murine messengers of NIK, IKKε and TAB2, predicted by the RNAhybrid algorithm. Lines indicate Watson-Crick base pairings, dots indicate wobble base pairs. Crosses denote positions of mutations introduced into the 3'UTR sequences in order to abrogate regulation of the luciferase 3'UTR reporters tested in Fig. 23. **(C)** Section of the alignment of NIK 3'UTR sequences of the indicated vertebrate species. The black frame highlights the conserved miR-155 seed-complementary site.

The respective 3'UTRs were fused to a *Renilla* luciferase reporter gene which was over-expressed in MEF cells along with the respective miRNA mimics, as described above (section 6.3). Compared to a control miRNA mimic, delivery of a miR-155 mimic significantly reduced the activity of the TAB2, IKKε and NIK reporters (Fig. 23A-C). Two consecutive point-mutations in the predicted miR-155 seed-pairing sites of the TAB2, IKKε and NIK reporters, respectively (Fig. 22B), abrogated regulation, which demonstrates direct targeting by miR-155 (Fig. 23A-C). Importantly, no specific suppression of the TAB2, IKKε and NIK reporters was observed upon delivery of a miR-146 mimic (Fig. 23A-C). It remained to be determined whether endogenous miR-155, induced upon LPS challenge of macrophages, impacts the activities of the TAB2, IKKε and NIK reporters. To this end, macrophages transfected with the intact or miR-155 binding-site deficient reporters were stimulated with 1 ng LPS / ml (miR-146 inducing stimulus; Fig. 18E, F) or 1000 ng LPS / ml (miR-155 inducing stimulus; Fig. 18D). Upon

24 h of LPS treatment reporter activity relative to control-treatment (no LPS) was determined. A significant repression of the wild-type TAB2, IKKε and NIK reporter constructs compared to the respective miR-155 binding-site mutant constructs was observed only at the miR-155 inducing stimulus of 1000 ng LPS per ml but not at the miR-146 inducing stimulus of 1 ng LPS per ml (Fig. 23D-F). These results suggest that the pro-inflammatory signal transducers TAB2, IKKε and NIK are selectively controlled by miR-155 (and not miR-146) during the macrophage inflammatory response.

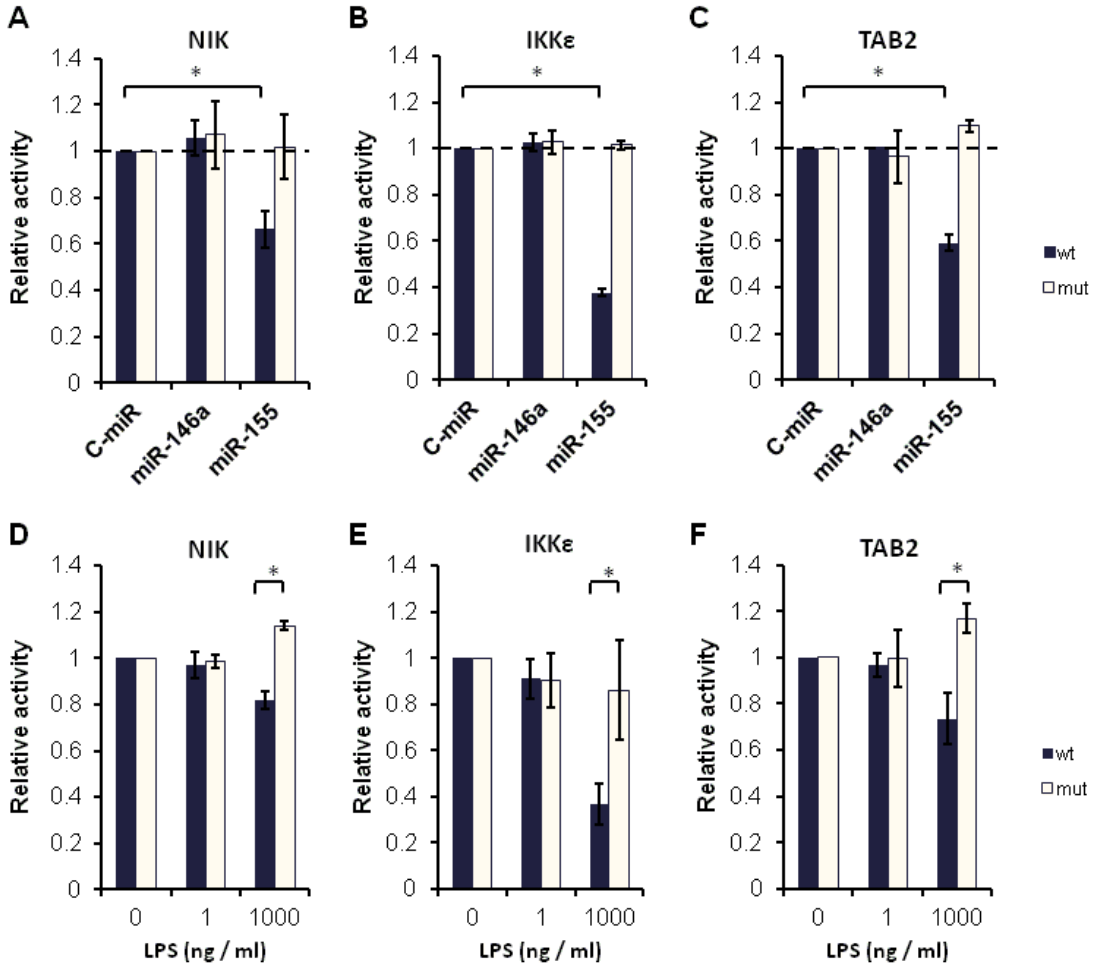

Figure 23: MiR-155 but not miR-146 targets NIK, IKKε and TAB2. (A-C) Activities of the indicated wild-type (wt) or miR-155 target-site mutant (mut) reporters were determined in MEF cells upon co-transfection of miR-146a or miR-155 mimics, compared to control mimic co-transfection. **(D-F)** The activities of the reporters introduced in (A-C) were tested in RAW264.7 macrophages upon stimulation with 1 or 1000 ng LPS / ml for 24 h compared to control-treatment (0 ng LPS / ml). Asterisks denote significant differences between the indicated pairs of samples (*P*-value < 0.05).

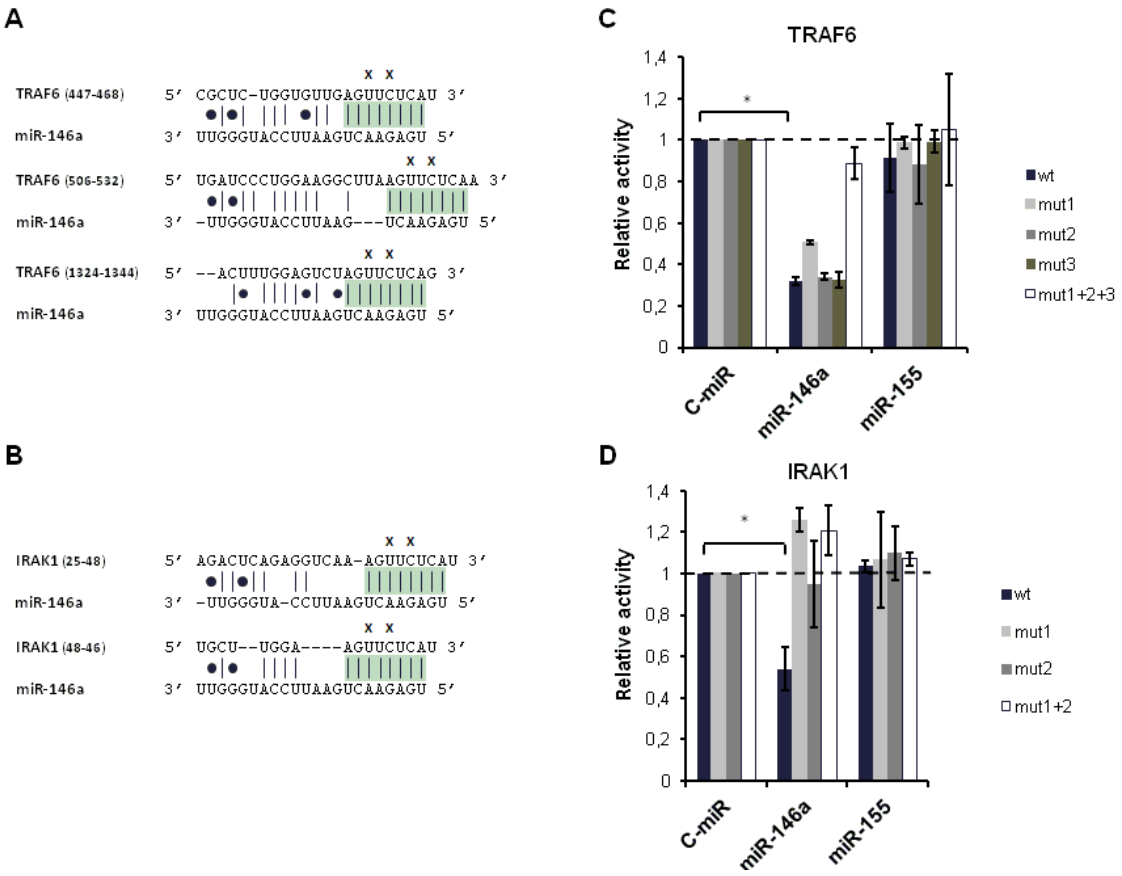

Figure 24: MiR-146 but not miR-155 targets TRAF6 and IRAK1 3' UTR reporters. (A, B) Binding sites of miR-146 within the 3'UTRs of the murine messengers of TRAF6 and IRAK1, predicted by the RNAhybrid algorithm. Lines indicate Watson-Crick base pairings, dots indicate wobble base pairs. Crosses denote positions of mutations introduced in order to abrogate regulation of luciferase 3'UTR reporters. **(D-E)** Activities of the indicated wild-type (wt) or miR-146 target-site mutant (mut) reporters were determined in MEF-cells upon co-transfection of miR-146a or miR-155 mimics, compared to control mimic co-transfection.. Asterisks denote significant differences between the indicated pairs of samples (*P*-value < 0.05).

To determine whether miR-155 and miR-146 act redundant or control distinct target profiles in the macrophage inflammatory response it remained to be addressed whether the known miR-146 targets IRAK1 and TRAF6 may also be repressed by miR-155. To this end IRAK1 and TRAF6 3'UTR reporters (Fig. 24A, B) were transfected into MEF cells. As expected (Taganov, et al., 2006) both reporters were readily repressed upon delivery of a miR-146 mimic; yet, the miR-155 mimic did not impact the reporter actvities (Fig. 24C, D). Introduction of two consecutive point-mutations into the predicted miR-146 target sites of IRAK1 and TRAF6 (Fig. 24A, B) rendered the reporters non-responsive to miR-146 (Fig. 24C, D).

Collectively, these results demonstrate that miR-146 and miR-155 control distinct targets in macrophage innate immune pathways. The selective control of signaling components involved in various pro-inflammatory pathways suggests that miR-155 constitutes a pervasive regulator of pro-inflammatory signaling, while miR-146 seems to be limited to control of TLR signal transducers (see Fig. 22A).

6.7 Control of the TNFα paracrine/autocrine signaling route by miR-155

The results described in the previous section, combined with the strict co-regulation of miR-155 with inflammatory marker genes (Fig. 17, 18), predicts a specific role of miR-155 in the macrophage pro-inflammatory response. This distinguishes miR-155 from miR-146, which is induced at sub-inflammatory TLR activity levels already and might control processes that precede inflammation. One of the specific targets of miR-155, TAB2, is a signal transduction kinase involved in both TLR and cytokine signaling (Kawai and Akira, 2006), (Fig. 22A). TAB2 reportedly mediates NFκB activation downstream of TNFα signaling, which is involved in sustaining the macrophage pro-inflammatory response to LPS through autocrine TNFα stimulation (Werner, et al., 2005). Therefore, control of pro-inflammatory TNFα signaling might represent a unique function of miR-155, justifying its strong association with the macrophage inflammatory response.

To test this hypothesis macrophages were transfected either with synthetic miRNA mimics or miRNA antisense-inhibitors of miR-155 and miR-146, followed by measuring NFκB activity induced by TNFα. To this end an engineered RAW264.7 macrophage clone (RAWBlue™) harboring a Secreted Embryonic Alkaline Phosphatase (SEAP) transgene under the control of an NFκB dependent promoter was used. NFκB activity was determined in cell culture supernatants by photometrical quantification of SEAP-induced substrate color shift. As a positive control the impact of miR-155 and miR-146 over-expression or inhibition on *S.* Typhimurium LPS induced NFκB activity was determined. LPS induced NFκB activaton has previously been reported to be negatively regulated by both miRNAs (Quinn and O'Neill, 2011), (section 4.3.2). Stimulation of RAWBlue™ macrophages with 1000 ng LPS / ml for 24 h induced the NFκB reporter by ~15-fold compared to a non-challenged control (Fig. 25A, C). Transfection of a control miRNA mimic did not impact on the LPS-induced reporter activity. However, miR-146 and miR-155 mimics (at 30 nM) significantly reduced reporter induction to ~9- and ~5-

fold (Fig. 25A). Simultaneous delivery of miR-146 and miR-155 mimics did not further reduce reporter activity below the activity observed for miR-155 mimic delivery, suggesting that miR-155 acts upstream of miR-146 in the control of LPS-induced NFκB activity (Fig. 25A). Transfection with miRNA inhibitors gave the reverse effect: miR-146 inhibition increased NFκB reporter activity to ~18-fold and inhibition of miR-155 increased the activity even further to ~23-fold at 10 nM of the inhibitor (Fig. 25C). Simultaneous inhibition of miR-146 and miR-155 with the same inhibitor concentration increased reporter activity to the level observed for miR-155 inhibition alone, confirming the results obtained from miR-146 and miR-155 mimic transfection (Fig. 25C).

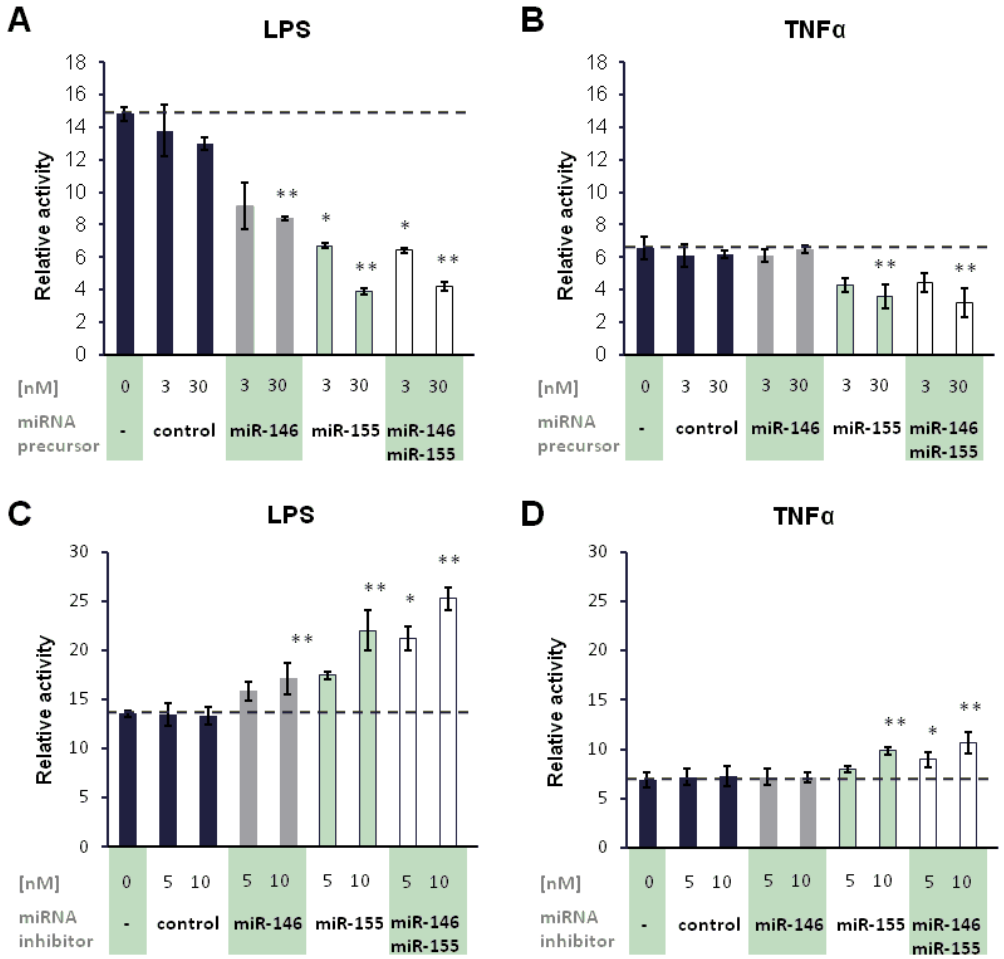

Figure 25: MiR-155 but not miR-146 restricts TNFα induced NFκB activity. (A, B) NFκB reporter activity relative to a non-stimulated control was determined 24 h post stimulation with LPS (A) or TNFα (B) in cells transfected with the indicated synthetic miRNA mimics (precursors). **(C, D)** Same experiment as an (A, B) but using the indicated miRNA inhibitors instead of mimics. * denotes a significant difference compared to the 3 nM (A, B) or 5 nM (C, D) controls, whereas ** denotes a significant difference compared to the 30 nM (A, B) or 10 nM (C, D) controls (*P*-value < 0.05).

When RAWBlue™ macrophages were stimulated with 200 ng recombinant TNFα / ml for 24 h, reporter activity increased by ~7-fold compared to a non-challenged control (Fig. 25B). In line with the above raised hypothesis of miR-155 controling TNFα signaling, miR-155 but not miR-146 mimics significantly decreased TNFα induced NFκB reporter activity to ~4-fold at 30 nM (Fig. 25B). Conversely, miR-155 but not miR-146 inhibition elevated the TNFα induced reporter activity to ~10-fold at 10 nM of inhibitor (Fig. 25D). In summary, the control of TNFα induced NFκB activity seems to represent a unique domain of miR-155 that distinguishes it from miR-146, while both miRNAs suppress LPS induced TLR4 signaling. Thus, the tight coupling of miR-155 to inflammation likely confers feedback control of pro-inflammatory cytokine signaling.

6.8 Roles of miR-146 and miR-155 in the control of macrophage LPS sensitivity

In contrast to miR-155, which stays strictly coupled to pro-inflammatory transcription and controls TNFα signaling, miR-146 is fully inducible at sub-inflammatory doses of *S.* Typhimurium LPS (Fig. 18). In line with this observation, miR-146 is even activated in endotoxin-tolerant macrophages upon challenge with attenuated *S.* Typhimurium (Fig. 17C, E). This predicts this miRNA to participate in the maintenance of LPS-hypo-responsiveness via control of TLR signal transducers IRAK1 and TRAF6 (Taganov, et al., 2006), (Fig. 24). This prediction is supported by the reported requirement of miR-146 for the maintenance of LPS tolerance via suppression of IRAK1 in intestinal epithelial cells (Chassin, et al., 2010).

To investigate the potential role of miR-146 in suppression of TLR signaling in endotoxin-tolerant macrophages, RAWBlue™ cells were transfected with either synthetic miR-146 or miR-155 mimics and challenged with a high dose of *S.* Typhimurium LPS (1000 ng / ml) or with recombinant TNFα (200 ng / ml) as a control. As expected, the response to LPS was strongly attenuated in endotoxin-tolerant compared to naïve macrophages (Fig. 26A). By contrast, TNFα stimulated NFκB activity, which is typically lower than LPS induced activity in naïve cells, proceeded normally in endotoxin-tolerant cells (Fig. 26B, C). Intruigingly, inhibition of miR-146 elevated LPS-stimulated NFκB reporter induction in endotoxin-tolerant cells from ~7 to ~12-fold but had no effect on TNFα induced reporter activity (Fig. 26B). By contrast, miR-155 inhibition did not affect the basal LPS stimulated reporter activity in endotoxin-tolerant

cells (Fig. 26B), which is in line with the weak induction of this miRNA under these conditions (Fig. 17C). However, miR-155 inhibition slightly elevated TNFα induced NFκB activity in endotoxin-tolerant cells (Fig. 26B), suggesting even weak induction of this miRNA to repress TNFα signal transduction. Interestingly, the over-expression of miR-146 in endotoxin-tolerant cells did not further reduce the LPS-stimulated reporter activity, suggesting the endogenous miR-146 to fully control TLR4 signal transduction (Fig. 26C). MiR-155 overexpression did not further reduce LPS sensitivity of endotoxin-tolerant macrophages either (Fig. 26C).

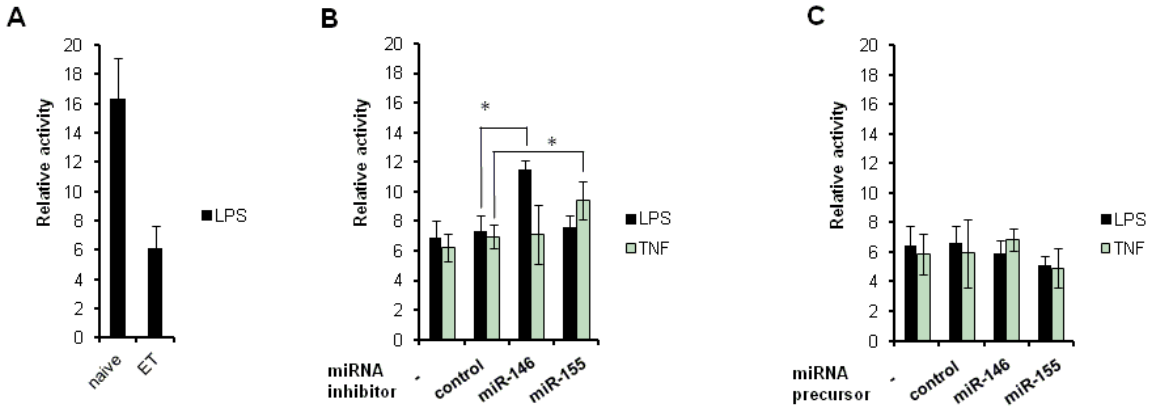

Figure 26: MiR-146 assists in the dampening of LPS sensitivity in endotoxin-tolerant RAW264.7 macrophages. (A) NFκB reporter activity in naive and endotoxin-tolerant RAWBlue macrophages, determined 24 h post stimulation with 1000 ng LPS / ml. **(B)** NFκB reporter activity in endotoxin-tolerant RAWBlue macrophages transfected with the indicated miRNA inhibitors and challenged with 1000 ng LPS or 200 ng TNFα per ml for 24 h. **(C)** Same experiment as in (B) but using synthetic miRNA mimics (precursors) instead of inhibitors. Asterisks denote significant differences between the indicated pairs of samples (*P*-value < 0.05).

In summary, these observations suggest that miR-146 plays a major role in the maintenance of endotoxin-tolerance and the prevention of premature activation of inflammation in macrophages. This involves negative control of LPS-induced NFκB activation, in line with previous reports on the role of miR-146 in negative control of TLR4 activity of intestinal epithelial cells (Chassin, et al., 2010).

6.9 MiR-155 responds to cytosolic microbial sensors of the NLR family

While endotoxin-tolerant macrophages keep TLR4 signaling and miR-155 suppressed, partially by the aid of miR-146 (Fig. 26), facultative intracellular wild-type *S.*

Typhimurium still activates a pro-inflammatory response (Fig. 17). This might involve an *S.* Typhimurium virulence factor or a host response that ensures induction of inflammation in response to the cytosolic appearance of bacteria even when TLR signaling is muted. The cytosolic NOD-like receptors (NLRs) of microbial peptidoglycan, NOD1 and NOD2, may activate NFκB independent of classical TLR-signal transducers IRAK1 and TRAF6 (Shaw, et al., 2010). NOD1 and NOD2 use a different signaling pathway than TLRs to mediate NFkB activation (Shaw, et al., 2010) and might therefore not be subject to negative control by miR-146 or other TLR-negative-regulators. Thus, NOD1 and NOD2 might provide a possible alternative route to miR-155 activation and inflammation in response to intracellular bacteria such as *S.* Typhimurium. Peptidoglycan is part of microbial cell walls and consists of short peptide chains that are linked to each other via N-acetylmuramic acid and N-acetyl-diaminopilic acid. The peptidoglycan degradation product γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) specifically activates the NOD1 receptor, while the degradation product N-acetyl-muramyl dipeptide (MDP) activates NOD2. A third peptidoglycan derivate, N-acetyl-muramyl-L-Ala-γ-D-Glu-meso-diaminopimelic acid (M-TriDAP) activates both receptors in the cytosol.

In order to study a possible role of the cytosolic NOD1 and NOD2 receptors in inflammatory response activation and thereby miR-155 induction in LPS hyposensitive cells, endotoxin-tolerant macrophages were transfected with peptidoglycan residues iE-DAP, MDP or M-TriDAP. Upon 24 h of incubation, NFκB activity (RAWBlue™ cells) and miR-155 expression (RAW264.7 cells) was determined. Successful induction of endotoxin-tolerance to was validated both at the level of NFκB reporter activity (Fig. 27A) and miR-155 expression (Fig. 27D). Cytosolic delivery of NOD1 agonist iE-DAP in endotoxin-tolerant macrophages did not activate the NFκB reporter or miR-155 expression, in line with NOD1 being hardly expressed in RAW264.7 macrophages[4]. NOD1/2 agonist M-TriDAP and NOD2 agonist MDP on the other hand mildly induced NFκB reporter (Fig. 27B) and miR-155 expression (Fig. 27E) by ~5-fold, similar to LPS challenge of endotoxin-tolerant cells (Fig. 27A, D). Strikingly, co-stimulation of endotoxin-tolerant macrophages with LPS and either M-TriDAP or MDP induced the NFκB reporter (Fig. 27C) and miR-155 expression (Fig. 27F) to similar levels as LPS stimulation of naïve macrophages (Fig. 27A, D). Thus NOD2 might initiate reactivation of

[4] RAWBlue™ technical datasheet (Invivogen Inc,)

inflammation in response to invading bacteria such as *S.* Typhimurium by terminating the inhibition of the LPS response and this also entails miR-155 induction.

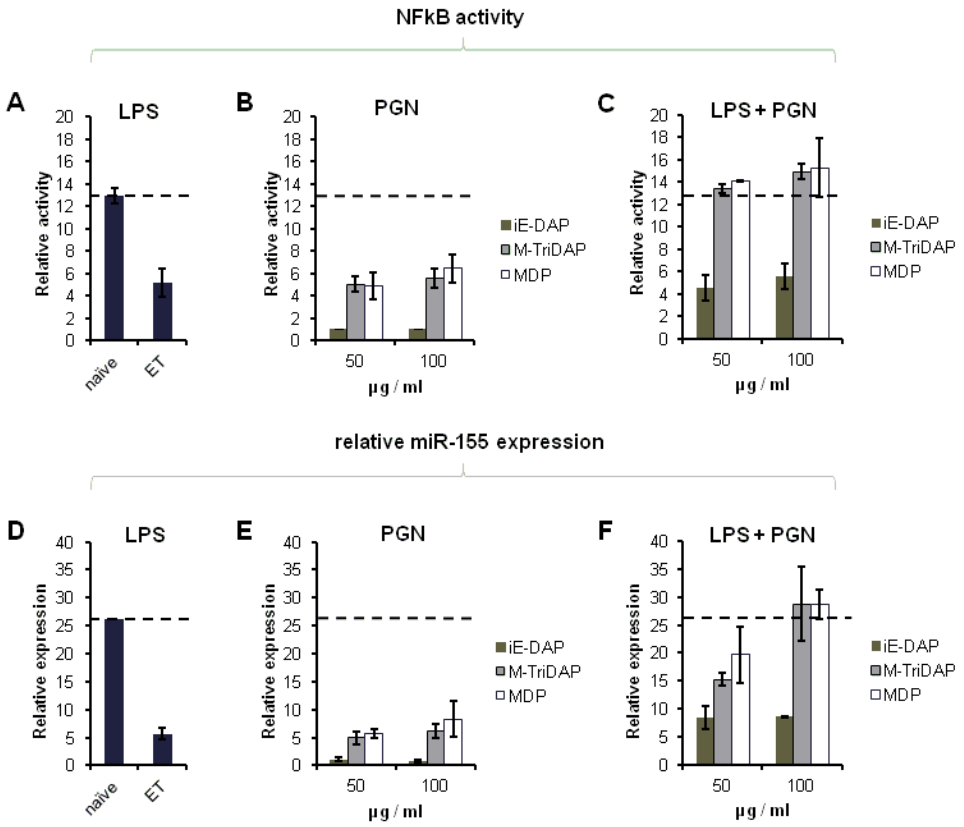

Figure 27: NOD2 stimulus restores LPS-triggered NFκB activity and miR-155 induction in endotoxin-tolerant macrophages. (A) NFκB reporter activity upon stimulation of naïve and endotoxin-tolerant (ET) RAWBlue™ cells with 1000 ng LPS / ml for 24 h, compared to mock-treated controls. **(B, C)** NFκB activity of RAWBlue™ cells upon stimulation with peptidoglycan derivates alone (B) or in combination with LPS (C) for 24 h, compared to mock-treated controls. **(D-F)** same experiment as in (A-C) but measuring miR-155 expression instead of NFκB activity in RAW264.7 cells by real-time PCR.

To examine whether miR-146 and miR-155 are involved in NOD2-, similar to TLR-triggered immunity, endotoxin-tolerant RAWBlue™ macrophages were challenged with either MDP alone or MDP and LPS simultaneously. The impact of miRNA overexpression and inhibition on NFκB reporter activity was determined. Delivery of a miR-146 mimic did not impact the ~5-fold MDP driven reporter induction; however, upon co-stimulation with MDP and LPS miR-146 repressed NFκB reporter activity to ~10-fold, compared to the ~15-fold induction upon control miRNA mimic delivery (Fig. 28A). A similar repression upon co-stimulation with MDP and LPS was observed upon miR-155 mimic delivery (Fig. 28A). Strikingly however, miR-155 elevated the reporter activity induced upon stimulation with MDP alone from ~5-fold to ~12-fold (Fig. 28A). This suggests that

miR-155 supports NOD2 mediated revocation of endotoxin-tolerance through a yet unknown pathway, before it negatively regulates the resulting inflammatory response. Inhibition of miR-155 did not alter MDP induced reporter activity, which is in line with this miRNA being hardly expressed in endotoxin-tolerant cells (Fig. 28B). Upon co-stimulation with MDP and LPS, however, inhibition of miR-155 elevated NFκB reporter activity from ~15 to ~25-fold compared to control-inhibitor delivery (Fig. 28B). This again supports the view that miR-155 functions in the inflammatory response as a co-regulated limiter.

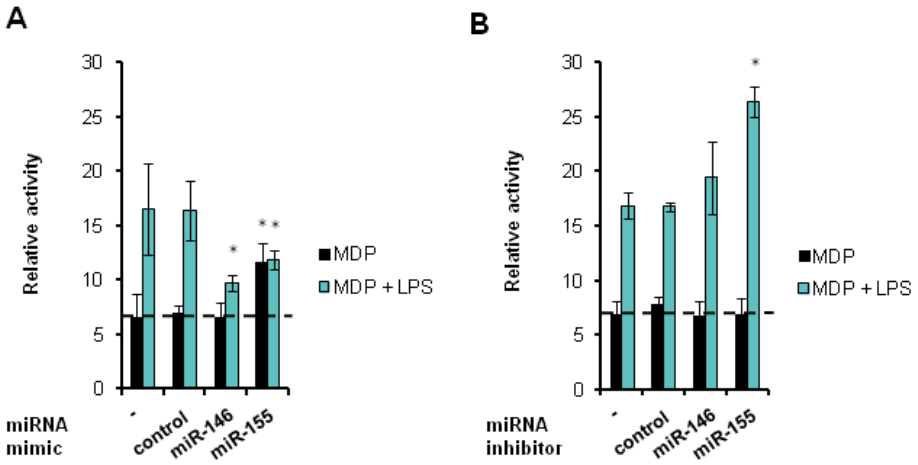

Figure 28: miR-146 and miR-155 modulate NFkB activity upon NOD2 pathway activation in endotoxin-tolerant macrophages. (A) NFκB reporter activity determined upon miRNA over-expression in RAWBlue™ cells stimulated with MDP alone or in combination with LPS for 24 h. **(B)** Same experiment as in (A) but using miRNA inhibitors instead of mimics. Asterisks denote significant differences compared to the respective control mimic or inhibitor treatments (P-value < 0.05).

The sum of the present results suggests that miR-146 is induced even in response to minute TLR4 stimuli in both naïve and endotoxin-tolerant macrophages to keep the cell from mounting a pre-mature inflammatory response. By contrast, miR-155 is mounted exclusively when the macrophage response proceeds to inflammation. Importantly, miR-155 may be activated upon cytosolic sensing of microbial peptidoglycan by the NLR NOD2, thus creating an alternative pathway to inflammation that may bypasses negative control by miR-146 when cells face an intracellular microbial pathogen (see Fig. 29).

7. Discussion

7.1 Major findings of the present study

The adequate responsiveness of mammalian immune cells to pathogenic threat is tightly regulated at the transcriptional and post-transcriptional level to avoid malfunctions, ranging from auto-aggressive and over-excessive immunity to immunodeficiency (Liew, et al., 2005). MiRNAs are increasingly being recognized as regulators of proper immune function (section 4.3). Yet, their specific roles in the innate immune response to living microbial pathogens had remained poorly characterized. Using a high-throughput sequencing approach this work identified miRNA regulations in murine and human host cells exposed to the facultative intracellular microbial pathogen model *S*. Typhimurium. Novel roles of miRNAs were revealed in *S*. Typhimurium challenged macrophages, a cell type that contributes to the first line of defense against pathogenic agents (section 4.1.2).

Intriguingly, the evolutionarily conserved let-7 family of miRNAs was found to be down-regulated in *S*. Typhimurium challenged epithelial cells and macrophages. In macrophages let-7 was down-regulated in an LPS-dependent manner via TLR4 and relieved repression of major cytokines IL6 and IL10 (see Fig. 29), which were identified as novel let-7 targets. These interleukins constitute key modulators of the systemic response to infection (see below). The genetic mechanism of let-7 down-regulation in microbially challenged macrophages remains to be revealed.

On the other hand, miR-146 and miR-155 were co-induced in *S*. Typhimurium challenged macrophages. Induction of both miRNAs reportedly depends on the pro-inflammatory transcriptional regulator NFκB (Quinn and O'Neill, 2011). Similar to let-7, miR-146 and miR-155 regulation upon *S*. Typhimurium challenge could be recapitulated with purified *S*. Typhimurium LPS. Despite their observed co-induciton in response to *S*. Typhimurium however, miR-146 was found to respond to even minute concentrations of LPS that failed to induce inflammation marker mRNAs or miR-155. Thereby miR-146 dampened the TLR4 response, protecting from pre-mature activation of inflammation and assisting in macrophage tolerance to LPS (see Fig. 29). MiR-155 was induced at higher doses of LPS, along with classical pro-inflammatory marker genes, and functioned as a limiter of the TNFα autocrine/paracrine signaling pathway that sustains the pro-inflammatory response to LPS (see Fig. 29). This suggests that certain miRNA functions

may only be revealed at a specific concentration of an environmental trigger, which might also hold true for miRNA controlled pathways that are unrelated to immunity.

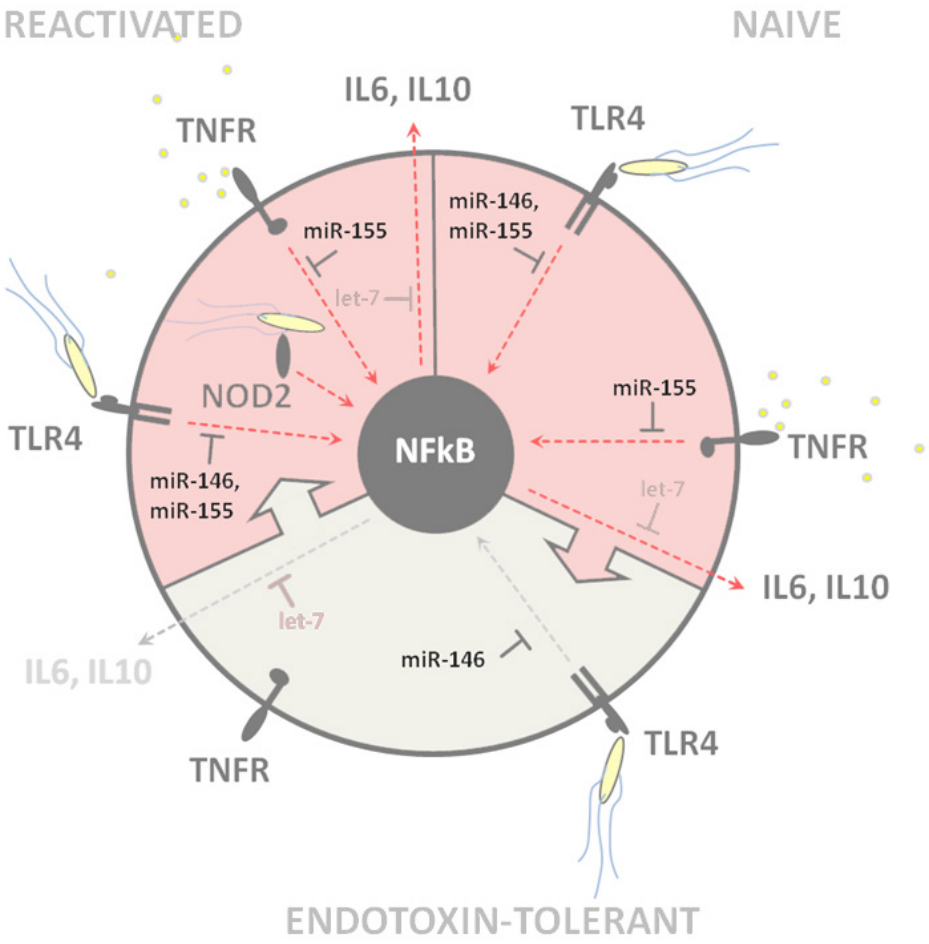

Figure 29: Model of miR-146, miR-155 and let-7 function in the macrophage response to microbial challenge. TLR4 stimulation of naïve macrophages co-activates miRNAs miR-146 and miR-155 to limit pro-inflammatory signaling and down-regulates let-7 to facilitate cytokine production. Upon progression into LPS-hyporesponsiveness (endotoxin-tolerance) miR-146 acts to keep LPS sensitivity low, while miR-155 stays silent. Let-7 down-regulation however might prepare the cell for a cytokine response already. Sensing of intracellular bacteria via NOD2 bypasses TLR4-hyposensitivity and reactivates the inflammatory response along with miR-155, which in turn functions to pervasively limit pro-inflammatory signaling pathways, such as TLR and TNFα signaling.

While the regulation of let-7, miR-146 and miR-155 may be triggered by plasmamembrane receptor TLR4 upon recognition of the LPS of *S*. Typhimurium, this pathogen may cross the plasmamembrane and stimulate cytosolic immune receptors as well. Importantly, an alternative route to the macrophage inflammatory response was identified that was initiated by the cytosolic peptidoglycan sensor NOD2 and induced miR-155 and NFκB activity. In contrast to TLR4, the NOD2 pathway was not negatively

regulated by miR-146. Therefore, this alternative route to inflammation might warrant responsiveness to cell-invasive pathogens such as *S*. Typhimurium even when LPS sensing is dampened by miR-146 (Fig. 29).

The examples provided by this work demonstrate that distinct miRNAs may control very different layers of cellular immunity with let-7 down-regulation promoting, miR-146 activation preventing and miR-155 induction limiting inflammatory gene expression in response to microbial threat.

7.2 Emerging functions of microRNAs in innate defense against microbes

7.2.1 The let-7 micoRNA family

Control of cytokine expression by let-7 miRNAs in microbially challenged macrophages. The present study demonstrates down-regulation of the let-7 family miRNAs in macrophages infected with *S*. Typhimurium or challenged with purified LPS. Intriguingly, let-7 down-regulation was found to relieve suppression of the major macrophage cytokines IL6 and IL10. These results were recently backed up by an independent study showing that let-7 down-regulation in LPS stimulated macrophages relieves IL10 repression (Liu, et al., 2011). The results presented here also show that despite of the arsenal of bacterial effectors that *S*. Typhimurium employs to manipulate host cellular pathways (section 4.5.3), let-7 down-regulation proceeds similarly in infected and LPS stimulated macrophages (Fig. 11). Even in sorted fractions of *S*. Typhimurium challenged macrophages let-7 was down-regulated to similar extents in cells that had internalized and cells that had probably faced but not taken up the pathogen (Fig. 10). This suggests that let-7 regulation is part of macrophage PAMP-triggered immunity and inert to subversion by the major microbial model pathogen *S*. Typhimuriuim.

Interestingly, the targets regulated by let-7 in macrophages, IL6 and IL10, may have antagonizing functions. IL6 mainly acts as a pro-inflammatory cytokine. In concert with the macrophage pro-inflammatory cytokines IL1 and TNFα, IL6 promotes systemic inflammatory symptoms such as fewer (Dinarello, 2009; Sundgren-Andersson, et al., 1998). If not counter-balanced, IL6-triggered inflammation may eventually result in septic shock (Biswas and Lopez-Collazo, 2009; Leon, et al., 1998). IL10 on the other hand functions to counteract the pathological effects of IL6 during microbial infection (Couper, et al., 2008). Therefore, the simultaneous derepression of both IL6 and IL10 in

microbially challenged macrophages might confer a balanced systemic response to the pathogen. Furthermore, IL6 and IL10 play a role in the initiation of T_{h2} like adaptive responses, which propel immunoglobulin production (section 4.3.5). In line with this, T_{h2} polarization of adaptive immunity has been reported for human patients with microbial infections, comprising a specific increase in serum levels of IL6 and IL10 but not of T_{h1} related cytokines such as IL-12 and IFN-γ (Tang, et al., 2011). Despite the established functions of IL6 and IL10 in anti-microbial immunity however, the physiological relevance of their regulation by let-7 in macrophages remains to be studied.

Let-7 miRNAs in epithelial PAMP-triggered immunity. Intriguingly, the here reported let-7 down-regulation in response to *S*. Typhimurium infection was not restricted to macrophages but occurred in epithelial cells (Hela229) as well (Fig. 6). Down-regulation of let-7 in Hela229 cells was observed both upon challenge with wild-type and SPI1/SPI2 deficient (attenuated) *S*. Typhimurium (Fig. 6C). This suggests that similar to macrophages let-7 down-regulation in epithelial cells is not actively promoted by the pathogen but rather reflects a host response to a microbial stimulus. This view is supported by a recent study on the gram-positive facultative intracellular microbial pathogen *L. monocytogenes* that similar to *S*. Typhimurium may cause food-born enteric or systemic disease. *L. monocytogenes* down-regulated let-7 miRNAs in infected intestinal epithelial cells (Caco-2) as part of a host response since both a wild-type strain and a virulence defect mutant triggered let-7 regulation (Izar, et al., 2012). However, the specific PAMPs that trigger let-7 regulation in response to both *S*. Typhimurium and *L. monocytogenes* infection remain to be identified. Previously, bile-duct epithelial cells had been shown to down-regulate let-7 in response to LPS (Chen, et al., 2007). However, Hela229 cells, used in the present study, are deficient in LPS sensing (Wyllie, et al., 2000). This indicates, that LPS is not the only possible *S*. Typhimurium PAMP triggering let-7 regulation in epithelial cells. Reportedly, intestinal epithelial cells largely depend on the flagellin-sensor TLR5 to mount a pro-inflammatory response to microbial pathogens (Gewirtz, et al., 2001). Thus, *S.* Typhimurium flagellin might constitute another possible PAMP triggering let-7 down-regulation in epithelia. Yet, both the host PRRs involved in let-7 down-regulation and the targets of this miRNA family in epithelial cells remain to be studied. Similar to macrophages, intestinal epithelial cells may produce the pro-inflammatory cytokine IL6 in response to *S.* Typhimurium challenge (Weinstein, et al., 1997). Moreover, studies in bile duct epithelial cells suggest that let-7 down-regulation

in response to LPS challenge might serve to feed-forward activate TLR4 and to relieve repression of the negative regulator of cytokine signaling CIS (section 4.3.4). Yet, the physiological significance of control of IL6, TLR4 and CIS by let-7 in the epithelial response to live microbial pathogens such as *S*. Typhimurium remains to be determined in *in vivo* infection models.

Emerging paradigms regarding let-7 funciton in anti-microbial defense. Collectively, the results presented here and by other recent studies suggest that down-regulation of let-7 miRNAs upon microbial challenge occurs in such different cell-types as macrophages and epithelial cells (Fig. 5, 6). Let-7 down-regulation seems to reflect a host immune program since infection with microbial model pathogens *S*. Typhimurium or L. *monocytogenes* triggers let-7 regulation similar to attenuated microbial strains or purified PAMPs (see above). The view of let-7 down-regulation being part of a general host defense program that is employed by various cell types is supported by recent reports on T cells. Both viral infection and T-cell receptor stimulation of $CD4^+$ T cells triggered down-regulation of let-7; similar to macrophages this relieved repression of IL10 (Kumar, et al., 2011; Swaminathan, et al., 2012). These results support recently anticipated modes of miRNA action in immunity. Generally, innate defense against microbial and viral pathogens in eukaryotes has been proposed to comprise induction of miRNAs that suppress negative regulators, and down-regulation of miRNAs that suppress positive regulators of defense (Ruiz-Ferrer and Voinnet, 2009). Indeed, the regulatory circuitry comprising let-7 and IL6 in macrophages provided a first example for down-modulation of a suppressor of a positive regulator of defense in mammals. Another example has recently been demonstrated in mice where *Mycobacterium bovis* or *Listeria monocytogenes* infection caused down-regulation of miR-29 in NK cells. This relieved repression of pro-inflammatory cytokine IFN-γ (Ma, et al., 2011), which indicates, that feed-forward control of defense promoting cytokines by miRNAs may reflect a common principle in innate immunity. Intriguingly, suppression of IL10 by let-7 establishes yet another paradigm of miRNA function in immunity that comprises down-regulation of a suppressor of a negative regulator of defense. Simultaneous control of IL6 and IL10 by let-7 in macrophages might serve to secure a balancing of pro- and anti-inflammatory responses to microbial pathogens, such as *S*. Typhimurium (see above). Yet, it remains to be tested whether IL6 and IL10 are simultaneously controlled by let-7

in other cell types such as epithelial cells as well and to which extend let-7 impacts the systemic response to *S*. Typhimurium in *in vivo* infection models.

7.2.2 MiR-146 and miR-155

Induction of miR-146 and miR-155 in anti-microbial immunity. Previous studies found miR-146 and miR-155 to be co-induced in the macrophage and monocyte response to microbial PAMPs (Monk, et al., 2010; O'Connell, et al., 2007; Taganov, et al., 2006). Analysis of host miRNA regulations by high-throughput sequencing confirmed co-induction of miR-146 and miR-155 upon live microbial infection of macrophages with wild-type and attenuated *S*. Typhimurium strains (Fig. 5). Despite the massive manipulation of cellular pathways by *S*. Typhimurium, regulation of miR-146 and miR-155 seemed to be inert to subversion by the pathogen (Fig. 8-11). In fact, cell sorting followed by miRNA expression analysis revealed that miR-146 and miR-155 regulation in macrophages that contained GFP-labeled *S*. Typhimurium or that had probably faced but not internalized the pathogen was similar to macrophages stimulated with purified *S*. Typhimurium LPS (Fig. 10, 11). The notion that major microbial pathogens may forgo manipulation of miR-146 and miR-155 induction in macrophages is supported by recent observations on miRNA regulation in macrophages challenged with the gram-positive microbial pathogen *L. monocytogenes*. Both a wild-type strain and a virulence-defect mutant of *L. monocytogenes* co-induced miR-146 and miR-155 to similar extends as purified PAMPs (Schnitger, et al., 2011). Yet, the cellular logic underlying miR-146 and miR-155 co-induction in microbially challenged macrophages was not well understood, especially as both miRNAs exert negative feedback control of TLR signaling (Quinn and O'Neill, 2011). Instead of being simply co-regulated and acting redundant miR-146 and miR-155 were found to have different induction thresholds in macrophages challenged with rising doses of *S*. Typhimurium PAMPs and to control different layers of the inflammatory response to microbes, as discussed in the following.

Control of macrophage TLR sensitivity by miR-146. The results presented here demonstrate that macrophages activate miR-146 in response to minute concentration of *S*. Typhimurium LPS, far below the concentrations required to trigger miR-155 or classical inflammation markers such as TNFα, IL6 or Ptgs2 (Fig. 18). Yet, LPS is not the only relevant PAMP involved in macrophage innate immunity to *S*. Typhimurium (Broz, et al.,

2012). TLR2 for instance is expressed on the macrophage plasmamembrane and senses microbial lipoproteins. When macrophages were stimulated with rising amounts of TLR2 agonistic lipoprotein, miR-146 was again activated in response to minute concentrations of PAMP, far below those required to fully induce miR-155 (Fig. 21). Previously, miR-146 has been shown to target the shared TLR signal transducers IRAK1 and TRAF6 (Taganov, et al., 2006). This study furthermore shows that IRAK1 and TRAF6 are exclusively targeted by miR-146 but not by miR-155 (Fig. 24). Therefore, dampening of TLR signaling in response to a given microbial PAMP might constitute a primary function of miR-146, functionally distinguishing it from miR-155. A recent report furthermore shows that activation of miR-146 by the PAMP LPS dampens the activity of TLR2, TLR4 and TLR5 via targeting of IRAK1 and TRAF6 (Nahid, et al., 2011). Thus miR-146 activation in response to minute PAMP doses might cross-control TLR activity and thereby protect from pre-mature inflammatory responses when microbial threat is low. Such a protective function of miR-146 is also supported by the observation that miR-146-knockout mice display an exaggerated inflammatory response to injected LPS, when compared to wild-type animals (Boldin, et al., 2011).

Once the primary response to microbial exposure peaks off macrophages may switch into endotoxin-tolerance, a state of TLR hypo-responsiveness that protects from inflammation induced pathologies (Biswas and Lopez-Collazo, 2009). The above discussed roles of miR-146 in feed-back suppression of TLR signaling render this miRNA a candidate involved in the maintenance of endotoxin-tolerance. Indeed, miR-146 was activated in LPS treated endotoxin-tolerant macrophages despite of a suppressed inflammatory response (Fig. 17). Inhibition of miR-146 partially restored the inflammatory response to LPS in endotoxin-tolerant macrophages (Fig. 26). These results are in agreement with previous reports on the role of miR-146 in dendritic cells and epithelial cells. Epithelial dendritic cell subsets constitutively express high levels of miR-146 to suppress TLR mediated inflammatory responses to commensal bacteria (Jurkin, et al., 2010). Similarly, Neonate intestinal epithelial cells express high levels of miR-146 to suppress the initiation of gut inflammatory responses to the establishing commensal microbiota (Chassin, et al., 2010). Collectively, miR-146 appears decrease TLR sensitivity in multiple cell-types to prevent from premature and inappropriate induction of inflammation.

Roles of miR-146 and miR-155 in response to intracellular bacteria. The above described observations suggest that miR-146 participates in the first line of innate defense against microbes by regulating TLR sensitivity via targeting of IRAK1 and TRAF6. Thereby miR-146 might protect from inflammatory pathologies in response to minute concentrations of PAMPs or to commensal microbes and promote endotoxin-tolerance. Yet, facultative intracellular pathogens such as *S*. Typhimurium may trigger cytosolic PRRs of the NLR family, that activate an inflammatory response independent of the miR-146 targets IRAK1 and TRAF6 (section 4.5.2). The cytosolic PRR NOD2 for instance recognizes degradation products of peptidoglycan, a microbial cell wall component. PAMP recognition by NOD2 mediates NFκB activation via the signaling molecules RIP2 and TAK1 (Shaw, et al., 2010). Unless NOD2 signaling components are also targeted by miR-146, this route could provide an alternative pathway to activation of the macrophage inflammatory response that is not subject to negative control by miR-146. Indeed, infection with cell-invasive, wild-type *S*. Typhimurium was found to abrogate endotoxin-tolerance in macrophages (Fig. 17). Yet, TLR-signaling seemed to be suppressed in these cells (partially via miR-146), as LPS or *S*. Typhimurium strains deficient in macrophage entry did not trigger an inflammatory response (Fig. 17, 27). Similar to wild-type S. Typhimurium infection, transfection a of NOD2 agonist abrogated endotoxin-tolerance. The resulting inflammatory response also reactivated miR-155, which functioned as a pervasive limiter of NFκB activity by targeting components of TLR signaling pathways and autocrine cytokine signaling (Fig. 23, 25). This suggests that the NOD2 pathway may safeguard the inflammatory response and activation of the co-regulated inflammation limiter miR-155, even when TLR signaling is mute. Besides *S*. Typhimurium also *L. monocytogenes* has been reported to reactivate the inflammatory response in endotoxin-tolerant macrophages and this involved the NOD2 pathway (Kim, et al., 2008). Thus, while miR-146 may establish TLR cross-tolerance to protect from inflammation induced pathologies, the NOD2 pathway might prevent from attenuation of defense against cell-invasive bacterial pathogens by reactivating the inflammatory response and miR-155. The latter limits macrophage pro-inflammatory signaling (Fig. 25) and might thereby protect from exaggerated inflammation. On the other hand miR-155 also promotes production of the pro-inflammatory cytokine TNFα (Kurowska-Stolarska, et al., 2011; Thai, et al., 2007). This might primarily serve to activate immune cells in the periphery as miR-155 restricts macrophage autocrine TNFα signaling.

In summary, these observations show that under conditions of attenuated TLR signaling host cells may mount different miRNA responses to extra- and intracellular bacteria, respectively. That is, invasion-deficient *S*. Typhimurium strains trigger miR-146, which helps to maintain TLR cross-tolerance, while miR-155 stays suppressed. Upon invasion by wild-type *S*. Typhimurium however miR-155 is reactivated along with the inflammatory response and this might involve intracellular activation of the NOD2 pathway. It remains to be tested however, whether the inflammatory response and miR-155 induction proceed in *S*. Typhimurium infected NOD2-deficient endotoxin-tolerant macrophages. So long, the possibility of *S*. Typhimurium effector proteins triggering inflammation reactivation in endotoxin-tolerant cells, possibly independent of NOD2, cannot be excluded (see section 7.3).

Engagement of miR-146 and miR-155 in macrophage miRNA networks. The present work demonstrates that miR-146 and miR-155, instead of simply being co-induced, respond to different concentrations of microbial PAMPs and control different layers of innate defense. While miR-146 is induced at even minute activity of TLR4 or TLR2 (Fig. 18, 20, 21) and dampens the TLR response, miR-155 is activated only when the inflammatory response is pronounced and functions as a pervasive limiter of pro-inflammatory signaling pathways (Fig. 17, 18, section 6.6-6.7). However, miR-146 and miR-155 are both engaged in feed-back loops; therefore, their function cannot be regarded in isolation. Rather, dampening of TLR signaling by miR-146 may prevent from induction of inflammatory genes and miR-155. Similar to miR-146, miR-21, another miRNA induced in microbially challenged macrophages, may affect miR-155 expression. MiR-21 suppresses PDCD4, a positive regulator of NFκB activity and negative regulator of IL10 (Sheedy, et al., 2009). Thereby, miR-21 may polarize the inflammatory response towards production of the anti-inflammatory cytokine IL10. The latter in turn suppresses not only the general macrophage pro-inflammatory response to LPS but also miR-155 induction (McCoy, et al., 2010; Monk, et al., 2010). Despite of miR-155 suppression however, miR-21 and miR-146 induction in LPS challenged macrophages pre-treated with IL10 proceed normally (McCoy, et al., 2010; Monk, et al., 2010). This supports the here presented view of miR-146 (and probably also miR-21) primarily associating with sub-inflammatory responses, while miR-155 expression is tightly coupled to inflammation. It remains to be tested, to which extend let-7 participates in this miRNA

network as its down-regulation in macrophages promotes production of miR-155 counteracting cytokine IL10, similarly to the proposed role of miR-21 in macrophages.

Since facultative intracellular bacterial pathogens such as *S*. Typhimurium may activate the NOD2 pathway it also remains to be addresses how miRNAs downstream of this pathway may influence each other. Intriguingly, TLR feed-back regulator miR-146 did not impact NOD2 activity while miR-155 at low levels enhanced and at high levels limited NFκB activity downstream of NOD2. These findings suggest that miR-146 might not restrict inflammation and miR-155 induction downstream of NOD2 activation, other than downstream of TLR activation. Therefore, certain miRNA networks may be PRR specific. It remains to be investigated whether the TLR-responsive miRNAs miR-21 and let-7 are involved in the NOD2 pathway and whether intracellular sensors of bacteria employ miRNAs that are not involved in the TLR response.

7.3 Roles of host microRNAs in microbial pathogenicity

7.3.1 Control of MicroRNA expression by microbial pathogens

S. Typhimurium employs a battery of effector proteins to manipulate host cellular pathways. This involves host cytosceletal rearrangements and subversion of endosomal maturation pathways, but also the manipulation of innate immune and cell-death associated pathways (McGhie, et al., 2009). In epithelial cells for instance the *S*. Typhimurium effectors SopE, SopE2 and SopB mediate activation of the NFκB transcription factor and propel production of epithelial pro-inflammatory cytokine IL-8 (Bruno, et al., 2009). Although epithelial cells may induce NFκB-responsive miRNAs such as miR-146 and miR-155 upon microbial challenge (Izar, et al., 2012; Liu, et al., 2010; Xiao, et al., 2009), no miRNAs were induced upon infection of epithelial cells with *S*. Typhimurium (Fig. 6). However, several immune pathways in Hela229 cells, used here as an established epithelial *S*. Typhimurium infection model, may be attenuated (Wyllie, et al., 2000). Therefore it remains to be addressed whether *S*. Typhimurium promotes NFκB dependent induction of miRNAs in alternative epithelial cell models. Challenge of Caco2 cells (a human intestinal epithelial cell line) with *L. monocytogenes* has recently been shown to trigger miR-146 (Izar, et al., 2012), thus providing a possible alternative infection model to investigate manipulation of miRNA regulation by microbial pathogens.

Although the NFκB responsive miRNAs miR-146 and miR-155 stayed mute in Hela229 cells infected with *S*. Typhimurium, several members of the let-7 miRNA family were down-regulated, similar to macrophages (Fig. 6). It is not clear yet, how let-7 down-regulation is achieved mechanistically and whether it depends on pro-inflammatory transcriptional programs similar to miR-146 and miR-155. However, down-regulation of let-7 miRNAs also seems to occur in other epithelial cell models upon stimulation with microbial PAMPs or living bacterial pathogens. *L. monocytogenes* infection of intestinal epithelial cells and LPS stimulation of bile-duct epithelial cells promotes down-regulation of let-7, which in turn controls of TLR4 expression and cytokine signaling (Chen, et al., 2007; Hu, et al., 2009; Izar, et al., 2012), (section 7.2.1). Enrichment of *S*. Typhimurium infected epithelial cells by cell sorting, which has been successfully applied to macrophages already (Fig. 10), is required to clarify whether *S*. Typhimurium may manipulate let-7 regulation. Intriguingly, the microbial pathogen *Helicobacter pylori*, which colonizes the human gastric epithelium, has recently been demonstrated to promote down-regulation of let-7 in epithelial cells via the secreted effector protein CagA (Matsushima, et al., 2011). This provides first indication that let-7 expression may indeed be subject to subversion by microbial pathogens.

Despite the open questions in epithelial cells, *S*. Typhimurium does not seem to manipulate the major innate immune associated miRNA regulations in naïve macrophages. Induction of miR-146, miR-155 and down-regulation of the let-7 miRNAs proceeded similarly in macrophages challenged with wild-type *S*. Typhimurium or with derivative mutants deficient in invasion (ΔSPI1), intracellular survival (ΔSPI2) or both (ΔSPI1/2), (Fig. 9). Even when macrophages were enriched by cell sorting for wild-type *S*. Typhimurium containing cells and miRNA expression was examined, no differences in miRNA regulations compared to cells that had faced but not internalized the pathogen were detected (Fig. 10). These results may suggest that regulation of macrophage miRNAs such as miR-146, miR-155 or let-7 is generally inert to subversion by microbial pathogens. Yet, other recent studies have shown that this is not the case. For instance, miR-155 has been reported to be induced in an NFκB dependent but TLR and NOD1/2 independent manner upon challenge with *H. pylori* (Koch, et al., 2012). Other than *S.* Typhimurium, *H. pylori* typically stays extracellular but may deliver virulence factors into host cells through a translocation machinery referred to as the type 4 secretion system (T4SS). While the major secreted *H. pylori* virulence factor CagA was not required for the PRR independent induction of miR-155, loss of the T4SS abrogated the *H. pylori*-

specific miR-155 regulation. This suggests that *H. pylori* may utilize its virulence machinery in a yet unknown manner to maintain miR-155 expression even when PRR signaling is mute. Furthermore, *Mycobacterium tuberculosis*, a human pathogenic bacterium that preferentially colonizes of the alveolar epithelia, has recently been shown to subvert miRNA expression in macrophages. Compared to the less virulent, opportunistic pathogen *Mycobacterium smegmatis*, *M*. *tuberculosis* suppressed miR-155 induction and promoted expression of another miRNA, miR-125 (Rajaram, et al., 2012). These reports demonstrate the principle ability of microbial pathogens to manipulate miRNA expression in macrophages, which may involve both suppression and promotion of host miRNA expression. While *S*. Typhimurium does not interfere with major host miRNA regulations it remains to be determined, whether this pathogen promotes or inhibits expression of host miRNAs that would not be regulated upon PAMP challenge. This could be achieved by enrichment of *S*. Typhimurium containing macrophages by cell sorting (see Fig 10), followed by global analysis of miRNA expression by high throughput sequencing.

While *S*. Typhimurium does not seem to manipulate the major host miRNA regulations in infected naïve macrophages, it remains unclear whether miR-155 activation in endotoxin-tolerant host cells is promoted by the bacterium. Wild-type *S*. Typhimurium but not the derivative virulence-defect strains or purified LPS activate miR-155 and the inflammatory response in endotoxin-tolerant macrophages (Fig. 17). While the NOD2 pathway may activate miR-155 and the NFκB dependent inflammatory response in macrophages (Fig. 27), it is not clear yet, whether *S*. Typhimurium triggers this pathway. For this reason, miR-155 expression and NFκB activity should be determined upon *S*. Typhimurium infection of endotoxin-tolerant macrophages deficient in NOD2 or down-stream signaling components such as RIP2. Independent of the NOD2 pathway, *S*. Typhimurium could alternatively promote miR-155 induction in endotoxin-tolerant macrophages via the secreted effectors SopE, SopE2 and SopB. Intriguingly, these effectors may activate Rho-GTPases to trigger an NFκB dependent inflammatory response via a yet poorly defined pathway (Bruno, et al., 2009). A potential promoting effect of *S*. Typhimurium secreted proteins on miR-155 expression could on the other hand be masked in naïve macrophages, which trigger pronounced miR-155 induction via TLR sensing of *S*. Typhimurium PAMPs (Fig. 9). Collectively, the present study suggests that *S*. Typhimurium does not subvert regulation of major host defense associated miRNAs in epithelial cells and naïve macrophages; yet this does not rule out the

possibility of promotion of host miRNA regulations (e.g. miR-155) by the pathogen, when host PAMP triggered immunity is suppressed.

7.3.2 Comparing the roles of host miRNAs in microbial virulence strategies

The present work suggests that the facultative intracellular microbial model pathogen *S*. Typhimurium does not actively interfere with major macrophage PRR induced miRNA regulations. It remains to be determined what distinguishes *S*. Typhimurium from other pathogens such as *H. pylori* or *M. tuberculosis* that do manipulate the macrophage miRNA response. Principally, the rapid dissemination of *S.* Typhimurium from the primary site of infection (Mastroeni, et al., 2009) might outcompete a necessity to extensively manipulate the local host immune response, as opposed to the rather long-term and locally restricted infestations by *H. pylori* or *M. tuberculosis* (Gomez and McKinney, 2004; Muller, et al., 2011). By timely escaping the host cell cytosol and targeting of distant sites of host tissue *S.* Typhimurium typically stays ahead of the local PAMP triggered inflammatory response (Mastroeni, et al., 2009).

In contrast to *S.* Typhimurium, the locally restricted infestations by *H. pylori* require a virulence strategy that involves suppression of excessive inflammatory responses of the infected gastric mucosa while maintaining mild inflammatory microenvironments that allow for continuous nutrient release. This is partially achieved via expression of PAMPs with reduced PRR affinity (Muller, et al., 2011) while simultaneously stimulating local macrophage miR-155 dependent survival pathways (Koch, et al., 2012). Macrophages are key to the maintenance of local inflammatory microenvironments (section 4.1.2), which *H. pylori* profits from; yet *S. Typhimurium* rather prefers to antagonize macrophage survival pathways in order to rapidly disseminate by escaping from the cytosol and the local immune response upon intracellular replication (Mastroeni, et al., 2009). Similar to *S*. Typhimurium, *L. monocytogenes* also forgoes manipulation of miRNA expression in macrophages (Schnitger, et al., 2011) and antagonizes macrophage survival to rapidly disseminate (Carrero, et al., 2004). Therefore *S.* Typhimurium or *L. monocytogenes*, other than *H. pylori*, might not benefit from promoting miR-155 expression.

M. tuberculosis has recently been reported to suppress induction of miR-155 in macrophages. Similar to *H. pylori* and in contrast to *S*. Typhimurium, *M. tuberculosis* establishes long-term infestations and has evolved to evade from host innate immunity.

Suppression of miR-155 by *M. tuberculosis* likely serves to prevent from its pro-inflammatory effects (Rajaram, et al., 2012), as miR-155 promotes the production of TNFα (Thai, et al., 2007). Therefore, similar to plant pathogen *P. syringae* (Navarro, et al., 2008), (section 4.4.2), *M. tuberculosis* suppresses a miRNA that acts as a positive regulator of defense. Other than *M. tuberculosis* however, *S. Typhimurium* might not profit from local attenuation of defense as it rapidly disseminates and to this end even employs immune cells recruited from the periphery as vehicles (Haraga, et al., 2008). As *H. pylori* promotes, *M. tuberculosis* suppresses and *S. Typhimurium* does not manipulate expression of miR-155 in macrophages it seems that for each individual pathogen it needs to be determined experimentally whether host miRNA expression changes conduce successful establishment of microbial infection, augment defense, or are dispensable for both.

A number of recent reports reveals miRNA regulations in mammalian host cells challenged with various microbial pathogens. All miRNAs found to be regulated upon challenge of host cells with *S*. Typhimurium (e.g. miR-21, miR-146, miR-155 and let-7) have recently been implicated in the host response to other microbial pathogens as well (Table 2). This suggests that the host miRNA response to *S*. Typhimurium is not pathogen-specific but rather reflects a basal host response to microbial challenge. However, several miRNAs with roles in microbial infection have not been investigated here as they were either not regulated upon *S*. Typhimurium challenge or poorly expressed in the investigated host cell models (Fig. 5 and 6). This includes the miR-372-373 cluster, miR-125, miR-149 and miR-29 (Table 2). At this stage however, it cannot be ruled out that these miRNAs play a role in *S*. Typhimurium infection of cell types or tissues not investigated in this study.

Table 2: miRNA regulations upon host cell infection with various microbial pathogens

	miRNA	Proposed function in response to the pathogen	Reference
		***Salmonella enterica* serovar Typhimurium**	
induced	miR-155	Negative feedback-control of **pro-inflammatory immune signaling** in macrophages; promotes **TNFα** production and **germinal center response**	This study; (Rodriguez, et al., 2007)
	miR-21	Unknown	This study
	miR-146a/b	Negative feedback control of **TLR4 signaling** in macrophages; prevents from premature responses to microbial LPS	This study; (Sharbati, et al., 2012)

decreased	let-7 family	Regulated upon infection or PAMP stimulation of macrophages and epithelial cells; decrease of let-7 expression promotes production of cytokines **IL6** and **IL10** in macrophages	This study; (Liu, et al., 2011)
		Helicobacter pylori	
induced	miR-155	Regulated in infected gastric biopsies, epithelial cells, T cells and macrophages; induced in macrophages in a *Helicobacter* T4SS dependent manner to activate **anti-apoptotic** pathways; promotes **Th1** and **Th17** responses and limits **inflammation** in infected mucosal tissues and epithelial cells.	(Fassi Fehri, et al., 2010; Koch, et al., 2012; Oertli, et al., 2011; Xiao, et al., 2009)
	miR-146	Regulated in infected gastric biopsies and cultured epithelial cells; negatively regulates **pro-inflammatory signalling**	(Liu, et al., 2010; Xiao, et al., 2009)
	miR-21	Unknown	(Belair, et al., 2011)
decreased	let-7 family	Regulated in gastric mucosal biopsies and cultured gastric epithelial cells in a *Helicobacter* CagA dependent manner; function unknown	(Matsushima, et al., 2011)
	miR-371-372-373 cluster	Regulated in gastric epithelial cells in a *Helicobacter* CagA dependent manner; decrease of miR-372 and miR-373 induced **cell cycle arrest**	(Belair, et al., 2011)
		Listeria monocytogenes	
increased	miR-155	unknown	(Izar, et al., 2012; Schnitger, et al., 2011)
	miR-146	unknown	(Izar, et al., 2012; Schnitger, et al., 2011)
	miR-125	unknown	(Schnitger, et al., 2011)
	miR-149	unknown	(Schnitger, et al., 2011)
decreased	miR-29	Regulated in NK and T cells upon infection to relieve repression of **IFN-γ** production	(Ma, et al., 2011)
	let-7a	unknown	(Izar, et al., 2012)
		Mycobacteria	
increased	miR-155	Induced in macrophages challenged with *M. avium*, *M. smegmatis* but suppressed by *M. tuberculosis* to circumvent the positive effect of miR-155 on **TNFα** production.	(Rajaram, et al., 2012; Sharbati, et al., 2012)
	miR-146	Induced by M. avium in macrophages; role in mycobacterial infection unknown	(Sharbati, et al., 2012)
	miR-125	Induced by *M. tuberculosis in macrophages*; negatively regulates **TNFα** production	(Rajaram, et al., 2012)
	miR-29	Elevated in the sputum of *M. tuberculosis* infected patients; unknown origin	(Fu, et al., 2011)
decreased	miR-29	Regulated in NK and T cells upon infection to relieve repression of **IFN-γ** production	(Ma, et al., 2011)

7.4 Conclusions and Outlook

The here presented results suggest that the host miRNA response to the facultative intracellular bacterium *S*. Typhimurium confers post-transcriptional control of important immune pathways and is is largely inert to manipulation by the pathogen. High-throughput sequencing of small RNAs revealed let-7 down-regualtion as the common denominator of infected murine macrophages and human epithelial cells. Macrophages furthermore induced the NFkB responsive miRNAs miR-146 and miR-155. The observations described here suggest that these miRNAs excert key functions in the macrophage inflammatory response to microbes by controling the expression of major host cytokines (let-7), regulating TLR sensitivity (miR-146) and limiting pro-inflammatory responses (miR-155). Yet, the present results also suggest that the host miRNA response may be plastic. That is, when macrophages switch into LPS hypo-responsivness (endotoxin-tolerance), which may protect from inflammation induced pathologies (Biswas and Lopez-Collazo, 2009), let-7 and miR-146 are still regulated in response to extracellular microbial stimuli, whereas miR-155 stays mute. By contrast, infection of endotoxin-tolerant macrophages with cell-invasive *S*. Typhimurium restores miR-155 induction along with the inflammatory response and this may involve the cytosolic sensor of microbial peptidoglycan, NOD2. These observations accentuate the use of a cell-invasive infection model. Intruigingly, recent reports show that live microbial infection of naïve macrophages with pathogens other than *S*. Typhimurium largely recapitulates the here presented miRNA regulations (Table 2). Thus, small RNA expression profiling of *S*. Typhimurium infected host cells by high-throughput sequencing proves itself succesfull in revealing miRNA regulations with significance to host interplay with various other microbial pathogens.

Besides the achievements, this work also poses a number of questions. For instance, let-7, which is down-regulated in microbially challenged host cells to relieve cytokine repression, is also a conserved suppressor of proliferation pathways in bilaterian metazoa (Boyerinas, et al., 2010). It remains to be studied whether let-7 down-regulation upon infection of macrophages not only propels cytokine expression but also macrophage proliferation, which might augment host defense. Let-7 might be involved in other macrophage post-transcriptional networks as well and impact the expression of different miRNAs. For instance, let-7 targets LPS sensor TLR4 (Chen, et al., 2007) and therefore it should be addressed whether let-7 regulation in microbially

challenged macrophages impacts expression of LPS inducible miRNAs such as miR-146 and miR-155.

Also, it is not clear yet, how let-7 is down-regulated in microbially exposed host cells. Induction of the pri-let-7 maturation inhibitor lin28 or of c-Myc, which suppresses pri-let-7 transcription (Wang, et al., 2011) could impact let-7 expression. Yet, mature miRNAs are rather stable with half-lifes of up to several days (Gantier, et al., 2011) and let-7 down-regulation in macrophages may occurr within a few hours (Liu, et al., 2011). Therefore it should be considered whether mature miRNA decay is involved in let-7 down-regulation; however, mature miRNA turnover in mammalian cells remains poorly understood. Intruigingly, HSV down-regulates host miRNAs by expressing instable target RNAs that mediate coupled decay of bound miRNA (section 4.4.1). As the mRNAs let-7 targets IL6 and IL10 carry instablility elements that accelerate turnover (Anderson, 2008) it should be considered whether let-7 down-regulation may involve coupled decay upon base bairing with its cognate target RNAs. It also remains to be studied how differential induction of miR-146 and miR-155 is controled in macrophages. Induction of both miRNAs requires the transcription factor NFkB (Quinn and O'Neill, 2011). Yet, miR-146 is sharply induced at sub-inflammatory doses of *S*. Typhimurium LPS while miR-155 expression increases gradually as a function of the provided LPS concentration (section 6.5). It has been postulated that most NFκB-dependent genes are induced gradually, due to non-cooperative promoter NFκB binding motifs, while an alternative all-or-nothing induction would require cooperative NFκB binding sites (Giorgetti, et al., 2010). It remains to be determined whether non-cooperative NFκB promoter elements drive the sharp induction of miR-146 at sub-inflamatroy LPS doses, and whether other genes in macrophages follow this pattern.

In addition to the cellular mechanisms of miRNA regulation, the target profiles of let-7, miR-146 and miR-155 require further investigation to understand their overall contributions to innate defense against microbial pathogens. To this end, pull-down and high-throughput sequencing of macrophage Ago2 bound mRNA may be applied, which has proven itself successful in other cellular systems already (Leung, et al., 2011). Given that an individual miRNA may affect the expression of hundreds of genes (Selbach, et al., 2008) further roles of the here presented miRNAs in innate immunity are expected to be revealed.

This study suggests that miRNAs control key pathways of innate defense against microbial pathogens and that miRNAs may be triggered by extra- and intracellular host

sensors of microbial PAMPs. As host cells may induce or down-regulate certain miRNAs only upon activation of specific PRRs, such as the cytosolic NLRs (Fig. 29), the miRNA response has to be regarded as plastic. This predicts that different microbial pathogens elicit different miRNA responses depending on which PAMPs they expose and whether they reside in extra- or intracellular host infection niches. It will be important to expand the current analysis of mammalian host miRNA expression changes to other cell types and other bacterial pathogens to understand whether miRNA directed defense is largely inert to manipulation or rather actively antagonized as part of a common microbial virulence strategy.

8. Materials and Methods

8.1 Cell culture, mice and bacterial strains

Hela229, RAW264.7 and MEF cells (all kindly provided by the group of Prof. Thomas Meyer, MPI-IB, Berlin), as well as RAWBlue™ cells (Invivogen) were cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal calf serum (Biochrom), 2 mM L-glutamine (Gibco), 1 mM sodium-pyrovate (Gibco), 0.1% β-mercapto-ethanol (Gibco), 1% penicillin/streptomicin solution (Gibco) at 37 °C, 5% CO_2 in a humidified atmosphere. If not specified differently, cells were seeded into 6-well or 24-well plates (Corning) at a density of 2×10^6 cells in 2 ml or 5×10^4 cells in 0.5 ml of medium respectively, 2 days prior to further treatment. Generally, experiments were carried out not before passage 5 and cells were discarded latest at passage 25. For long-term storage cells grown into passage 5 were suspended in fetal calf serum, supplemented with 10% cell-culture grade DMSO (Sigma) and stored in liquid nitrogen.

Bone marrow derived macrophages (BMDMs) were generated using bone marrow of the hind-legs of female wild-type or $TLR4^{-/-}$ C57BL/6 mice between 8 and 12 weeks of age. Isolated bone marrow was washed twice with RPMI, supplemented with penicillin/streptomycin by pelleting (centrifugation at 250 g for 5 min) and resuspending. Cells were then resuspended in X-vivo 15 medium (Lonza) supplemented with 10 % fetal calf serum and 10% L929 differentiation supernatant (kindly provided by the group of Prof. Dr. Stefan Kaufmann, MPI-IB, Berlin), seeded into 10 cm Petri-dishes at a concentration of 5×10^6 cells in 10 ml of medium. At day 3 of incubation, 5 ml of fresh medium were added and at day 5 cells were disattached by scraping and seeded into 24-wells for further treatment at day 7.

For endotoxin-tolerance induction RAW264.7 or RAWBlue™ macrophages were pre-exposed to heat-killed *S. typhimurium* at an MOI of 10 (Fig. 17) or 1000 ng LPS per ml (Fig. 24-26) for 20 h in a 75 cm^2 flask at ~70 % confluency. Cells were subsequently washed with PBS (four times) and cultured in fresh medium for three additional days. Afterwards cells were seeded into 6-well plates (Fig. 17) or 24-well plates (Fig. 24-26) two days prior to further treatment (assuring a total gap of five days between removal of the initial microbial stimulus and onset of experimental treatment).

Throughout this study *Salmonella enterica* serovar Typhimurium strain SL1344 (JVS-1574) is referred to as wild-type. The attenuated virulence mutants lacking the SPI1 (JVS-0405) or the SPI2 island (JVS-1103) were kindly provided by Susanne Paetzold (Paetzold, et al., 2007) and Karsten Tedin (Hansen-Wester, et al., 2004), respectively. The strain lacking both the SPI1 and the SPI2 island (JVS-3614) was constructed by P22 phage transduction of the ΔSPI1 strain with a lysate of the ΔSPI2 strain. The GFP-expressing wild-type *S.* Typhimurium strain (strain JVS-3858) was described recently (Papenfort, et al., 2009). Enteropathogenic E. coli (EPEC) strain E2348/69 and E. coli K12 strain DH5α were kindly provided by Dr. Jan-Peter Böttcher (MPI-IB, Berlin).

8.2 Bacterial infection and PAMP or cytokine stimulation of host cells

Host cells were seeded into 6-well plates two days prior to the infection. Bacterial strains were grown over-night in L-broth (LB) medium at 37° C (shaking), diluted 1:100 in fresh LB medium and grown until an OD of 2. Bacteria were pelleted (12000 rpm, 2 min) and redissolved in RPMI medium without antibiotics, fully supplemented as described above (section 8.1). Upon 1:100 dilution in RPMI medium the OD was determined again and a bacterial suspension was generated containing 1×10^7 cells per ml, assuming 1×10^9 bacteria per ml at an OD of 1. RAW 264.7 and RAWBlue™ cells were infected at an MOI of 1 while Hela229 cells were infected at an MOI of 10 after the cell culture supernatants had been exchanged for RPMI free of antibiotics. Upon centrifugation at 250 g for 10 min the cell-culture dishes were incubated at 37° C, 5% CO_2 in a humidified atmosphere. Thereafter the supernatants were replaced for RPMI medium containing 50 µg of gentamicin per ml in order to kill all remaining extracellular bacteria. Following 30 min incubation, the culture supernatants were finally replaced for RPMI medium containing 30 µg gentamicin per ml. In order to quantify bacterial host cell invasion and intracellular replication, cell cultures were washed twice with PBS buffer (Gibco) and then lysed by the addition of 1 ml PBT buffer (PBS supplemented with 0.1% of Triton X-100 [Sigma]) at the respective time-points. Lysates were plated on LB-agar and bacterial colony forming units (CFUs) were counted upon over-night incubation at 37° C. The obtained numbers were compared to the CFU counts of the plated input solution used for the initial infection of host cell cultures (see above). Results of CFU assays shown in

this study are presented as mean-values and standard-deviations of three independent experiments.

For stimulation of host cells with microbial PAMPs or recombinant TNFα cells were seeded into 6-well plates (Fig. 11) or 24-well plates (Fig. 18-26) two days prior to the exposure. Purified *Salmonella enterica* serovar Typhimurium LPS (Sigma, #L6143) and FliC (Alexis; #ALX-522-028) or TNFα (R&D, # 410-MT) were added into the cell-culture supernatants at the indicated final concentrations. The peptidoglycan derivates iE-DAP (Invivogen, # tlrl-dap), M-TriDAP (Invivogen, # tlrl-mtd) and MDP (Invivogen, #tlrl-mdp) were delivered into the host cell cytosol via lipofection using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For co-stimulations with the peptidoglycan derivates, LPS or TNFα were added into the cell-culture supernatants right upon addition of the lipofection mix.

8.3 Ectopic expression and inhibition of microRNAs

Ectopic expression of miRNA was achieved by transfection of Pre-miR™ synthetic miRNA precursor molecules (Ambion, control: # AM17110, mmu-miR-146: equimolar mix of # PM10722 and # PM10105, mmu-miR-155: # PM13058; has-let-7a: # PM10050; hsa-let-7d: # PM11778). For inhibition of miRNA activity cells were transfected with FITC-labeled miRCURY LNA miRNA-specific knockdown probes (Exiqon; control: # 199004-08; miR-146: equimolar mix of # 41063-08 and # 410066-08; miR-155: # 411222-08; hsa-let-7a: # 410017-08; hsa-let-7d: # 410020-08). Transfections of RAW264.7 and RAWBlue macrophages and MEF cells were carried out overnight, 24 h upon seeding into 24-well plates using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For inhibition of let-7 miRNA activity in RAW264.7 macrophages (Fig. 16E-G) cells were enriched for the FITC-labeled knock-down probe containing populations by cell-sorting (see below).

8.4 Northern blotting

Total RNA from control-treated or infected host cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For northern blot analysis of miRNA expression 25 µg of total RNA per lane (lyophilized, re-dissolved in 15 µl of gel-

loading buffer II [Ambion] and boiled for 2 min) were separated on 15 % poly-acrylamide (PAA), 8 M urea gels at 250 V in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0). 2 µl of 5' [γ-^{32}P]ATP-labeled Decade size marker (Ambion, prepared following the manufacturer's instructions) were loaded into a separate pocket of the gel. Upon separation RNA was transferred from the gel onto a Hybond™ XL membrane (Amersham) in a wet-blot chamber in 0.5 × TBE buffer at 50 V for 50 min. Upon transfer RNA was UV-crosslinked at 120 mJ per cm^2 using a Biolink UV Crosslinker (PeqLab). RNA species of interest were detected using [γ-^{32}P]ATP labeled DNA or LNA probes. Labeling of probes was carried out using 1 µl of oligo-nucleotide (25 pmol), 3 µl of [γ-^{32}P]ATP (Perkin Elmer), 1 µl of polynucleotide kinase (NEB) and 3 µl of provided buffer A (NEB) in a 20 µl reaction. Upon incubation for 1 h at 37 °C free nucleotides were removed from the radioactive probe using Sephadex G-25 spin colums (Amersham Biosciences) according to the manufacturer's instructions. For detection of miRNA, [γ-^{32}P]ATP labeled pre-designed LNA oligos (Exiqon; mmu-let-7a: #39510-00; mmu-miR-21: #39103-00; mmu-miR-146a: #39466-00; mmu-miR-155: #39471-00 and hsa-miR-1308: 21304-00) were hybridized over knight at 62 °C in Rapid-Hyb hybridization buffer (GE Healthcare) and the hybridized membrane was washed two times for each 15 min at 62 °C with 15 ml 2 × SSC solution (150 mM sodium chloride, 17 mM sodium citrate, pH 7.0). Detection of sno202 normalization control was carried out similarly but using a DNA probe (JVO-5873, 5′-AAGTACTTTCATCAAGTCAGTACAGC-3′) at a hybridization and washing temperature of 42 °C. [γ-^{32}P]ATP signals were detected using an FLA 3000 Phosphoimager system (Fuji).

8.5 Real-time PCR

For real-time PCR experiments, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. All real-time PCR experiments were carried in triplicates on a CFX96 system (Biorad) and expression-changes were calculated according to the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001). Real-time PCR results shown in this study are presented as mean-values and standard-deviations of three independent experiments.

Quantitative real-time PCR analysis of mature miRNA expression changes was carried out using TaqMan miRNA assay kits according to the manufacturer's instructions

(Applied Biosystems; hsa-miR-98: ID 000577; hsa-*let-7a*: ID 000377; hsa-let-7d: ID 002283; hsa-miR-146b: ID 001097; hsa-miR-146a: ID 000468; mmu-miR-155: ID 001806). Analysis of miRNA primary transcript expression changes was carried out using TaqMan pri-miRNA assay kits (Applied Biosystems; pri-miR-146a: ID Mm03306349_pri; pri-miR-146b: ID Mm03308117_pri; pri-miR-155: ID Mm03306395_pri). Both for mature and primary miRNA expression changes snoRNA202 served as a reference control (Wong, et al., 2007) and was detected using a pre-designed TaqMan kit (Applied Biosystems; snoRNA202: ID 001232) following the manufacturer's instructions.

Real-time PCR analysis of mRNA expression changes was carried out using Power SYBR Green RNA-to-CT™ 1-Step kits (Applied Biosystems) according to the manufacturer's instructions. DNA oligos (Sigma) used for SYBRGreen real-time PCR are listed below:

Table 3: SYBRGreen® real-time PCR oligos used in the present study

Oligo name	Sequence (5' →3')	Purpose
JVO-5397	TCTGATGATGTATGCCACCATC	Ptgs2 detection (sense)
JVO-5398	TTGATAGTCTCTCCTATGAGTATGAGTCTG	Ptgs2 detection (antisense)
JVO-7229	ATGAAGTTCCTCTCTGCAAGAGA	IL6 detection (sense)
JVO-7230	TCTGTTGGGAGTGGTATCCTC	IL6 detection (antisense)
JVO-5395	ACCACGCTCTTCTGTCTACTGAAC	TNFα detection (sense)
JVO-5396	TGAGAAGATGATCTGAGTGTGAGG	TNFα detection (antisense)
JVO-7403	GATCTGGCACCACACCTTCT	β-actin detection (sense)
JVO-7404	GGGGTGTTGAAGGTCTCAAA	β-actin detection (antisense)

8.6 High-throughput sequencing

Cells from control treated and infected samples were stored in RNAlater reagent (Qiagen) and send to Vertis Biotech AG (Freising, Germany) for RNA extraction, size-fractionation (recovery of the 19-29 nt RNA fraction) and subsequent cDNA library preparation. Libraries were sequenced on a Roche FLX 454 platform. Obtained cDNA reads were aligned to the human or murine mature miRNA reference sequences (miRBase version 14) using the R-package mirMap454, kindly provided by Dr. Jörg

Hackermüller (Fraunhofer Institute for Cell Therapy and Immunology, Leipzig). The mapping algorithm has been described in reference (REF: Schulte et al.). The number of reads obtained per individual miRNA was normalized to the total number of cDNA reads of the respective library. Only miRNAs that were represented by at least 0.1 % of all reads in one of the libraries were considered for further analysis.

8.7 Microarrays

Global analysis of mRNA expression changes in RAW 264.7 macrophages was performed by dual-colour hybridization of total Cy3 or Cy5 labeled RNA preparations on mouse whole genome G4122F 4 × 44k multipack microarrays (Agilent Technologies). Dye-specific effects were detected by colour-swapping (Churchill, 2002). RNA labelling was performed using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. Microarrays were scanned using a G2565CA microarray laser scanner (Agilent Technologies). Raw images were analysed using the Image Analysis/Feature Extraction software G2567AA (Version A.9.5, Agilent Technologies) and the extracted data were analysed using the Rosetta Resolver Biosoftware, Build 7.1 (Rosetta Biosoftware). Fold changes of individual genes were calculated based on the relative intensity values obtained from the control and infection sample dual color hybridizations. Heatmap representation of infection-induced mRNA expression changes was achieved by log2 transformation of mRNA fold changes using the 'Cluster' Software ' and visualization of the result table using the 'Treeview' software (Eisen, et al., 1998). The microarray data presented here are deposited in NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and can be accessed through the GEO Series accession number (GSE27703).

8.8 Reporter assays

NFκB activity was determined by analysis of Secreted Embryonic Alkaline Phosphatase (SEAP) activity in the supernatants of control-treated or agonist-challenged RAWBlue™ macrophages. SEAP activity in culure supernatants was determined using QuantiBlue™ reagent (Invivogen) according to the manufacturer's instructions and transparent 96-

well flat bottom plates (Corning). Colorimetric read-out was performed on an Ascent Plate Reader (Thermo Scientific) using the 630 nm filter.

Luciferase 3'UTR reporter assays were carried out using the pSICHECK-2™ plasmid (Promega) harboring a *Renilla* reporter luciferase and a firefly reference luciferase. Target validations were carried out in MEF cells by co-transfecting the respective 3'UTR reporter plasmid (250 ng) with synthetic miRNA precursors (30 nM) as described in under "Ectopic miRNA-expression and -inhibition". 24 h post transfection cells were lysed with "passive lysis buffer" (Promega) according to the manufacturer's instructions. 3 µl of cell lysate per sample were transferred into a well of a white, non-transparent 96-well flat-bottom plate and upon addition of 40 µl Beetle Juice (PJK GmbH) firefly luciferase activity was recorded on a Wallac 1420 Victor3 plate reader (Perkin Elmer). In a second step, 40 µl of pH 5.0-adjusted *Renilla* Juice (PJK GmbH) were added and *Renilla* luciferase activity was recorded. *Renilla* (reporter) activity counts were devided by firefly activity counts to obtain a value for reporter activity corrected for variances in transfection efficiency. Luciferase 3'UTR reporter assays in RAW264.7 macrophages were performed as described for MEF cells, however allowing the macrophages to recover for 5 days upon plasmid transfection before resuming with further experimental treatment.

Results of all reporter assays shown in this study are presented as mean-values and standard-deviations of three independent experiments.

8.9 ELISA

Cytokine production by RAW264.7 cells was measured using 50 µl aliquots of fresh cell culture supernatants. Cytokine concentrations (pg / ml) were determined using the OptEIA Mouse IL6 ELISA kit (BD Pharmingen) or the Quantikine Mouse IL10 ELISA kit (R&D Systems) according to the manufacturer's instructions. For IL6 ELISAs cell culture supernatants were diluted 1:10 in fully supplemented RPMI medium in order not to exceed the dynamic range of the assay kit. ELISA plates were read out on an Ascent Plate Reader (Thermo Scientific). All ELISA results shown in this study are presented as mean-values and standard-deviations of three independent experiments.

8.10 Cell sorting

RAW264.7 macrophages infected with GFP-expressing *S.* Typhimurium or transfected with FITC-labeled LNA miRNA knockdown-probes (Exiqon) were washed twice with PBS and harvested by scraping. The cell suspension in PBS was filtered through a MACS pre-separation filter (Miltenyi Biotec) to exclude large cell aggregates. Cells were then sorted using a FACS Aria II or a FACS Aria III platform (BD Biosciences). Intact cells were gated based on size (forward-scatter) and granularity (side-scatter) and GFP- or FITC-positive cells were detected by plotting the autofluorescence (PE-chanel) against the specific fluorescence (FITC-chanel) of this population. This identified a distinct population of cells in the infected or LNA-containing cells that was absent in the repective control treatments. This population was separated from the fluorophore negative population of cells using the gates shown in Fig. 10.

8.11 Cloning

For the construction of pSICHECK-2 3'UTR luciferase reporter constructs the respective DNA sequences were amplified from mouse cDNA using the primer pairs listed in table 4. For cDNA generation total RNA was extracted from female BL/6 mouse macrophages, using TRIzol reagent (Invitrogen). Reverse transcription was carried out using a Superscript II reverse transcription kit (Invitrogen) and Oligo(dT) primers (Invitrogen) according to the manufacturer's instructions. PCR amplification of 3'UTR sequences (extracted from current ENSEMBL annotations) was carried out using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer's instructions. PCR amplification products were cloned into the pSICHECK2™ reporter plasmid (Promega) using the *Xho*I and *Not*I restriction sites of the multiple cloning site. Restriction enzymes and buffers as well as DNA ligase were purchased from Fermantas and cloning was done according to the manufacturer's instructions. Ligation reactions were transformed into chemically competent *E. coli* TOP10 (Invitrogen) and bacterial colonies containing the desired insert were screened by colony PCR. Briefly, a bacterial colony was picked, suspended in 100 µl of sterile water and boiled for 5 min. 5 µl were introduced into a PCR reaction using Taq-Polymerase (NEB) according to the supplier's protocol. The pSICHECK-2™ sense sequencing oligo and the respective antisense 3'UTR amplification oligo served as colony PCR primers. Endotoxin-free preperations of

plasmids were obtained using Highspeed Midi Prep™ kits (Qiagen) according to the manufacturer's protocol. 3'UTR reporter constructs were verified by capillary sequencing (SMB services). Mutation of predicted miRNA binding sites was carried out by amplifying the respective reporter plasmid by regular PCR (15 cycles) using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific) and an oligor pair with ~12-16 nt 5' overlap harboring the desired mutations. Upon PCR amplification 1 µl of *Dpn*I (NEB) was added to the reaction to degrade PCR template DNA during 3 h of incubation at 37° C. 5 µl of the reaction were then transformed into competent *E. coli* TOP10 (Invitrogen) and introduction of point-mutations was validated by sanger sequencing (SMB services). Where necessary, DNA was run on analytical gels with an agarose content of 0.8% in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). DNA was stained for visualization under UV light by 15 min incubation in a TAE bath supplemented with ethidium bromide (Roth).

Table 4: cloning and mutagenesis oligos used in the present study

Oligo name	Sequence (5' →3')	Purpose
3'UTR reporter oligos (cloning adapters in lowercase):		
JVO-4410	atcggactcgagTGCGTTATGCCTAAGCATATC	IL6 (sense, XhoI site)
JVO-4411	tccgatgcggccgcAAATATAATATAATTTATTTGTTTGAAGACAGTCT	IL6 (antisense, NotI site)
JVO-4414	atcggactcgagAACACCTGCAGTGTGTATTGAG	IL10 (sense, XhoI site)
JVO-4415	tccgatgcggccgcCGAATAAGATCCATTTATTCAAAAT	IL10 (antisense, NotI site)
JVO-8224	atcggactcgagTCCTGTCTTCCACCACCAG	NIK (sense, XhoI site)
JVO-8225	tccgatgcggccgcTCGTCATTCCAAATTTTATTGCTTATAG	NIK (antisense, NotI site)
JVO-8250	atcggactcgagGCTCCCTGGGGGTTCACA	IKKε (sense, XhoI site)
JVO-8251	tccgatgcggccgcAGGAAAACAGAAAAGGCGATGTATTTATT	IKKε (antisense, NotI site)
JVO-7490	atcggactcgagGCCAAAGGCCCCGCC	TAB2(sense, XhoI site)
JVO-7491	tccgatgcggccgcTTAGAATATAAGTTTTTTTAATTTTTATAAATAA CTTATCTGTTACAAAGATA	TAB2 (antisense, NotI site)
JVO-7494	atcggactcgagTTTGTTCACTCTGACAAATCCCTC	IRAK1 (sense, XhoI site)
JVO-7495	tccgatgcggccgcTAGGTTGGCAATGGAGTAAAATTATC	IRAK1 (antisense, NotI site)
JVO-7496	atcggactcgagCGTCCATGTACTTGTGTTCAAAAAC	TRAF6 (sense, XhoI site)
JVO-7497	tccgatgcggccgcTGCAAATAAATCCTTTATAAAAATACTCCTGT	TRAF6 (antisense, NotI site)

3'UTR reporter miRNA target site mutagenesis (mutated nucleotides in red):		
JVO-4540	AGTTGCAAGACATGAATTGCTAATTTAAATATGTT	IL6 (sense)
JVO-4541	TCATGTCTTGCAACTTATACATTCCAAGAAACC	IL6 (antisense)
JVO-7688	GGAAAACCTCGTTTGCCAACATCTCCGAAATATTTATC	IL10 #1 (sense)
JVO-7689	GATAAATATTTCGGAGATGTTGGCAAACGAGGTTTTCC	IL10 #1 (antisense)
JVO-7690	CTCCGAAATATTTATCCAACATGACCAACAAGTTCCCATTCTATTTATTC	IL10 #2+3(sense)
JVO-7691	GAATAAATAGAATGGGAACTTGTTGGTCATGTTGGATAAATATTTCGGAG	IL10 #2+3 (antisense)
JVO-8790	ATTGTAGCCTGAAGTTCACTGTGAATC	NIK (sense, A →C, T→G)
JVO-8791	TGAACTTCAGGCTACAATGAGTCCTAAG	NIK (antisense, A →C, T→G)
JVO-8788	AAACTAGCCTGACTTTGACTGTCC	IKKε (sense, A →C, T→G)
JVO-8789	AAAGTCAGGCTAGTTTGTTGGC	IKKε (antisense, A →C, T→G
JVO-8713	TAGAAAGAAGTAATGAAATGAAACT	TAB2 (sense, C →A, T→G)
JVO-8714	TTTCATTACTTCTTTCTACCTTATG	TAB2 (antisense, C →A, T→G
JVO-8701	TCAAAGGTATCATGCTTGGAAGTT	IRAK1 #1 (sense, T →G, C→A
JVO-8702	AGCATGATACCTTTGACCTCTGAGT	IRAK1 #1 (antisense, T →G,
JVO-8703	TGGAAGGTATCATAGTGTGCAAAC	IRAK1 #2 (sense, T →G, C→A
JVO-8704	ACTATGATACCTTCCAAGCATGAGA	IRAK1 #2 (antisense, T →G,
JVO-8705	GTTGAGGTATCATTTAGTTGACTCCTG	TRAF6 #1 (sense, T →G, C→A
JVO-8706	TAAATGATACCTCAACACCAGAGC	TRAF6 #1 (antisense, T →G
JVO-8707	CTTAAGGTATCAAGTACTCCCTCCTCTATAG	TRAF6 #2 (sense, T →G, C→A
JVO-8708	TACTTGATACCTTAAGCCTTCCAGG	TRAF6 #2 (antisense, T →G
JVO-8709	GTCTAGGTATCAGGGAGCCCTAC	TRAF6 #3 (sense, T →G, C→A
JVO-8710	TCCCTGATACCTAGACTCCAAAGTAC	TRAF6 #3 (antisense, T →G

8.12 Statistical tests

Differences between two observations were routinely tested for significance using a two-tailed Student's *t*-test, assuming either equal variances or in the case of observations that had been made relative to a control that participates in the test, assuming unequal variances. *P* values < 0.05 were considered indicative of statistical significance.

9. Abbreviations

BCR = B cell receptor

BMDM = bone marrow derived macrophage

ceRNA = competing endogenous RNA

CFU = colony forming unit

DMSO = dimethylsulfoxid

dsRNA = double-stranded RNA

ET = endotoxin-tolerant

FACS = fluorescence activated cell sorting

g = × gravity

h = hour

HKS = heat-killed *S*. Typhimurium

iE-DAP = γ-D-glutamyl-meso-diaminopimelic acid

kb = kilobase

lincRNA = long intergenic non-coding RNA

LNA = locked nucleic acid

lncRNA = long non-coding RNA

LPS = lipopolysaccharide

mb = megabase

MDP = N-acetyl-muramyl dipeptide

min = minute

ml = milliliter

mM = millimolar

MOI = multiplicity of infection

M-TriDAP = N-acetyl-muramyl-L-Ala-γ-D-Glu-meso-diaminopimelic acid

ncRNA = non-coding RNA

ng = nanogram

NLR = NOD-like receptor

nM = nanomolar

nt = nucleotide

OD = optical density

ORF = open reading frame

PAMP = pathogen associated molecular pattern

PRR = pattern recognition receptor

pg = picogram

pi = post infection

PBS = phosphate buffered saline

RNAi = RNA-interference

ROS = reactive oxygen species

rpm = rounds per minute

SCV = *Salmonella*-containing vacuole

siRNA = small interfering RNA

ssRNA = single-stranded RNA

TLR = Toll-like receptor

TCR = T cell receptor

UTR = untranslated region

T3SS = type three secretion system

SPI1 = *Salmonella* pathogenicity island 1

SPI2 = Salmonella pathogenicity island 2

vs = versus

µg = microgram

µl = microliter

µM = micromolar

10. References

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11. Appendix

11.1 Selbständigkeitserklärung

Ich versichere hiermit, dass ich die vorliegende Dissertation unter Kenntnis der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 01.09.2005 selbständig und ausschließlich unter Verwendung der angegebenen Hilfsmittel und Hilfen erarbeitet und verfasst habe. Desweiteren ist die vorliegende Arbeit zu keinem früheren Zeitpunkt in einem Promotionsverfahren angenommen oder als ungenügend bewertet worden.

Berlin, den 18.10.2012

Leon Schulte

11.2 Contributions of third parties to the present work

Prof. Dr. Jörg Vogel (IMIB, Würzburg, Germany) has supervised the present work, contributed conceptually and assisted in experimental design throughout the study.

Dr. Ana Eulalio, as a post-doctoral scientist in the Vogel Group (MPI-IB, Berlin, Germany), participated in the here reported work on let-7 mediated control of cytokine IL6 and IL10 expression (section 6.1-6.4), assisted in molecular cloning and luciferase reporter assays and has carried out experimental replicates depicted in Fig. 14A, B and Fig. 15.

Alexander J. Westermann, as a PhD student in the Vogel Group (IMIB, Würzburg, Germany), has participated in the present work on miR-146 and miR-155 regulation (section 6.5-6.9) by assisting in the construction and validation of luciferase reporters.

Dr. Hans-Joachim Mollenkopf, responsible for the microarray core facility at the MPI-IB (Berlin, Germany), has assisted in the design of mRNA microarray experiments and conducted the microarray hybridizations and subsequent statistical evaluations.

High-Throughput Sequencing of small RNA was carried out at the sequencing core facility of the MPI-MG (Berlin, Germany) under the guidance of Dr. Richard Reinhardt.

Preparation of cDNA libraries for High-Throughput Sequencing was carried out by Vertis Biotech AG, (Freising, Germany).

11.3 Publications and scientific meetings

11.3.1 Publications

Differential activation and functional specialization of miR-155 and miR-146 in innate immune sensing (2012); **Schulte LN**, Westermann AJ, Vogel J. Nucleic Acid Research (in press)

The mammalian miRNA response to bacterial infections (2012); Eulalio A, **Schulte LN**, Vogel J. RNA Biology 9(6):742-750

Analysis of the host miRNA response to *Salmonella* uncovers the control of major cytokines by the let-7 family (2011); **Schulte LN**, Eulalio A, Mollenkopf H-J, Reinhardt R, Vogel J. EMBO Journal 30:1977-1989

Analysis of A to I editing of miRNA in macrophages exposed to *Salmonella* (2010). Heale BS, Eulalio A, **Schulte LN**, Vogel J, O'Connell MA. RNA Biology 7(5):116-122

11.3.2 Scientific meetings

Attending / poster-presentation:

- SIROCCO Annual Meeting 2007, Berlin, DE
- RNA Annual Meeting 2008, Berlin, DE
- ESF Meeting 2009, Granada, ES
- SIROCCO Annual Meeting 2009, Cambridge, UK
- ASM General Meeting 2009, Aix-en-Provence, FR
- IMBA Microsymposium on Small RNAs 2010, Vienna, AT
- SIROCCO Annual Meeting 2010, Heidelberg, DE
- NGFN Annual Meeting 2010, Berlin, DE
- 6th Berlin Genetics Workshop 2010, Berlin, DE

Speaker at scientific meetings:

- 13th Regional Drosophila Meeting 2007, Berlin, DE
- SIROCCO/RIGHT Young Scientists Symposium 2008, Cambridge, UK
- SIROCCO Annual Meeting 2008, Hinxton, UK
- NGFN Workshop 2010, Würzburg, DE
- NGFN Workshop 2011, Munich, DE
- RNA Annual Meeting 2011, Kyoto, JP